Functional analysis of W- and WT-boxes from the promoter of the *WRKY30* gene in response to microbe-associated molecular pattern (MAMP)

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List of abbreviations

2,4-D	2,4-dichlorophenoxyacetic
3-AT	3-amino 1,2,4-triazoles
4-MU	4-methylumbelliferone-4
A. thaliana	*Arabidopsis thaliana*
ABA	Abscisic acid
APS	Ammonium
ATP	Adenosine triphosphate
B. cinerea	Botrytis cinerea
BAP	6-Benzylaminopurine
BP	Base pairs
BSA	Bovine serum albumin
CaMV	Cauliflower mosaic virus
Carb	Carbenicillin
cDNA	Complementary
Col-0	Columbia 0
CRM	*cis*-regulatory module
°C	Celsius
DAMP	Damage-associated molecular pattern
ddH_2O	Double distilled water
dH_2O	Deionized water
DNA	Deoxyribonucleic
dNTP	Deoxy-nucleoside triphosphate
DTT	Dithiolreitol
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ETI	Effector-triggered immunity
GUS	β-glucuronidase
h	Hours
His	Histidine
Kan	Kanamycin

LB	Luria Broth
Leu	Leucine
LRR	Leucine-rich repeats
LUC	Luciferase
MAMP	Microbe-associated molecular pattern
MES	2- (N-Morpholino) ethanesulfonic acid
Min	Minute
MS	Murashige and Skoog
MTI	MAMP-triggered immunity
MUG	4-methylumbelliferyl-β-D-glucuronide
NB	Nucleotide binding
NM	Nanometer
OD	Optical density
P. crispum	Petroselium crispum
P. syringae	Pseudomonas syringae
PAMP	Pathogen-associated molecular pattern
pBT10	pBT10GUS-d35SLUC
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
pH	Potentia hydrogenii
PR	Pathogenesis-related
PRRs	Pattern recognition receptors
PTI	PAMP-triggered immunity
PTI	PAMP-triggered immune
RLKs	Receptor-like kinases
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription PCR
S. cerevisiae	Saccharomyces cerevisiae
SA	Salicylic acid

SAR	Systemic acquired resistance
Sec	Second
Taq	Thermus aquaticus
TATA	TATA box (minimal promoter)
T-DNA	Transfer DNA
TF	Transcription factor
TFBS	Transcription factor binding site
TRIS	Tris (hydroxymethyl) aminomethane
Trp	Tryptophan
TTSS	Type three secretion system
UV	Ultraviolet
Y1H	Yeast One-Hybrid
ND	Not determine
g	Gravitational force
NO	Nitric oxide
ETS	Effector-triggered susceptibility
R	Resistance
HR	Hypersensitive
JA	Jasmonic acid
ET	Ethylene
EF-TU	Elongation factor Tu
Flg22	Flagellin 22
AtPep1	*Arabidopsis thaliana* plant elicitor peptide 1
PGN	Peptidoglycan
LPS	Lipopolysaccharides
OG	Oligogalacturonides
FLS2	Flagellin sensitive2
BRI1	Brassinosteroid-Insensitive 1
BAK1	BRI1-associated receptor kinase 1 (BAK1)
BIK1	Botrytis-induced kinase 1
MAPK	MAP kinases

PEPR1	PEP receptor 1
BEST	Binding Site Estimation Suite of Tools
SDS	Sodium dodecyl sulphate
TE	Tris-EDTA
EMSA	Electrophoretic mobility shift assay
NT	Nucleotide
Rifa	Rifampicin

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1. Introduction

1.1 Plant-pathogen interactions

For the defense against pathogens, the interaction between plant and pathogens is inevitable. Since plants, as different from animal creatures, have no adaptive immune system, they are completely dependent on their innate immune response. This response occurs in every infected cell and systemic signals are transferred to the surrounding cells to alert more distant tissues (Nürnberger et al., 2004). Whether plant-pathogen interaction leads to resistance or disease of the plant depends on the immune system of the plant and the virulence strategy of the phytopathogen. The interaction between plant and pathogen may be regarded as a kind of evolutionary process. This occurs, for obtaining a selective advantage, alternately for adaptation of the plant and the pathogen. The four stage model shown in Figure 1 illustrates this evolutionary process (Bent and Mackey, 2007).

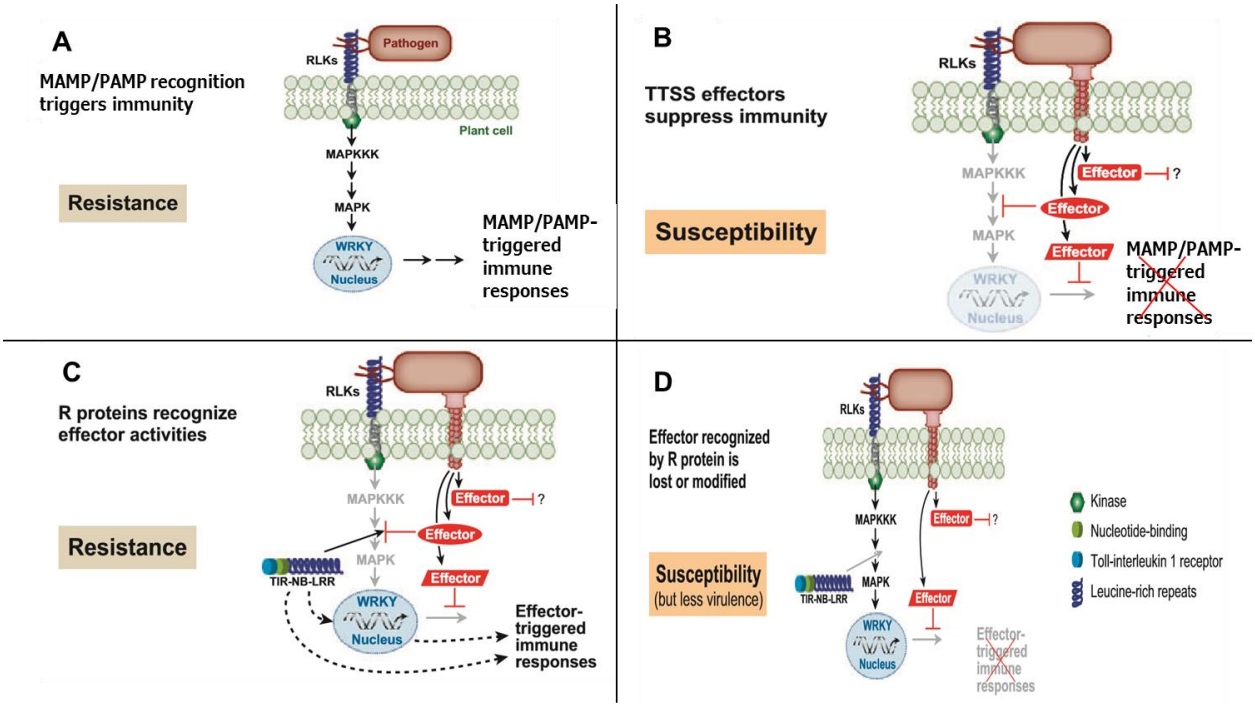


Figure 1. Four-stage model for plant-pathogen interaction.

(A) Plants recognize MAMPs/PAMPs (such as bacterial flagellin) by extracellular receptor-like kinases (RLKs) and trigger MAP kinase cascades leading to transcriptional reprogramming mediated by plant WRKY transcription factors. This process activates MAMP/PAMP-triggered immue responses. (B) Bacterial pathogens use the type III secretion system (TTSS) to deliver multiple

effector proteins that target host proteins and suppress basal immune responses, allowing significant accumulation of bacteria in the plant apoplast. (C) Plant resistance proteins (R proteins, such as a TIR-NB-LRR protein) recognize effector activity resulting in effector-triggered immune responses. (D) Pathogens avoid R gene-mediated defenses by modifying or eliminating the effector(s) that trigger those defenses (modified from Bent and Mackey, 2007).

In the first step, PAMPs (pathogen-associated molecular pattern) or MAMPs (microbe-associated molecular pattern) are recognized by cell membrane receptors of the plant. These highly conserved structures are usually located on the cell surface of microorganisms and are specific to a particular pathogen. Examples of MAMPs are bacterial flagellin, fungal chitin or peptidoglycans of bacterial cell walls. The first membrane receptors (PRRs; pattern recognition receptor) which recognize the MAMPs are receptor-like kinases (RLKs). Approximately 2.5% of the genome of *Arabidopsis thaliana* code for about 600 RLKs. In addition, a significant receptor subfamily for plant immune defense are the LRR kinases (Leucine rich repeat kinases) (Fritz-Laylin et al., 2005; Shiu, S. and Bleecker, 2001; Shiu, S. H. and Bleecker, 2003). Of them, more than 200 LRR kinases were found which are potential MAMP receptors (Jones and Dangl, 2006). Plants recognize MAMPs by RLKs and trigger MAP kinase cascades leading to transcriptional reprogramming mediated by plant WRKY transcription factors. This process activates PAMP/MAMP-triggered immue responses (Bent and Mackey, 2007; Glowacki et al., 2011; see Figure 1A). This comprises a MAP-kinase cascade which changes the expression pattern in the cell nucleus so that a resistance response is induced. This resistance is the basal or innate immune response of plants, which is relatively weak and slow, compared to the ETI (see below). The resistance responses include the production of, for example, reactive oxygen species (ROS), nitric oxide (NO) or phytoalexins, the closure of the stomata, or the deposition of lignin and callose in the cell wall (He et al., 2007). For triggering a pathogen response, exceeding a MAMP threshold is required, which is intended to prevent a response to non-pathogenic microorganisms (Bent and Mackey, 2007). Through different PAMPs/MAMPs or various analogous phytopathogens, convergent defense responses may be triggered (He et al., 2007).

There are specialized components of microorganisms (effectors) which inhibit active parts of the basal immune response (PTI or MTI) (Figure 1 B). This is called effector-triggered susceptibility (ETS). A pathogen usually contains 20 to 100 virulence effectors, which are

actively released into the host cell. Bacterial effectors, such as those from *Pseudomonas syringae* strains are transferred through the type three secretion system (TTSS) into the cytoplasm of the plant cell (Heath, 2000; Jones and Dangl, 2006). It is shown that mutants in the TTSS make colonization of the plant host impossible (Mudgett, 2005). Since the PTI or MTI is reduced by the influence of effectors, the pathogen can easier multiply and thus cause more damages to the plant (Jones and Dangl, 2006).

In response to the virulence factors, the plant resistance (R) genes were developed which can perceive the presence of certain effectors directly and indirectly (Figure 1 C). Many of these R-genes have a leucine rich repeat (LRR) domain and a nucleotide binding domain (NB). The LRR domain regulates the activity of the protein and recognizes the effector, whereas the NB domain is responsible for signal transduction and ATP hydrolysis (Belkhadir et al., 2004). After the effector is detected by the LRR domain, there is a much stronger response of the plant, resulting in effector-triggered immunity (ETI). ETI is a hastened and amplified PTI response, resulting in disease resistance and a hypersensitive cell death response (HR) at the infection site (Jones and Dangl, 2006). Most often the ETI results in a hypersensitive reaction in which there is a rapid, local cell death and which leads to signal transmission to surrounding or distant cells. The plant is resistant to further pathogen attacks and also protected even to other pathogens (Bent and Mackey, 2007; Glowacki et al., 2011).

Pathogens can evade R protein recognition by eliminating or modifying their effectors. The corresponding phytopathogen can be no longer recognized by the R protein. However, the pathogen is less virulent (Bent and Mackey, 2007; Figure 1D).

1.2 WRKY transcription factors

WRKY transcription factors (TFs) play an important role in the plant resistance response (Figure 1; Buscaill and Rivas, 2014). WRKYs are one of the largest families of transcriptional regulators in plants with 74 members in *Arabidopsis* (*Arabidopsis thaliana*), 109 members in rice (Oryza sativa), and more than 100 member in soybean or popular (Bakshi and Oelmüller, 2014; Eulgem and Somssich, 2007; Pandey and Somssich, 2009; Ross et al., 2007; Rushton et al., 2010).

The WRKY domain contains the highly conserved WRKYGQK peptide sequence at its N-terminus end and the zinc-finger-like motifs Cys(2)-His(2) or Cys(2)-HisCys. WRKY factors

bind to the TTGACT/C W-box *cis*-element in the promoter of their target genes (Agarwal et al., 2011; Rushton et al., 2010).

WRKY proteins can be classified on the basis of both the number of WRKY domains and the features of their zinc-finger-like motif. WRKY proteins with two WRKY domains belong to group I, whereas most proteins with one WRKY domain belong to group II. The WRKY domains of both group I and group II members have the same type of finger motif, potential zinc ligands (C–X4–5–C–X22–23–H–X1–H). WRKY proteins having only one WRKY domain but different patterns of zinc finger motifs are categorized into group III. Instead of a C2–H2 pattern, group III WRKY domains contain a C2–HC motif (C–X7–C–X23–H–X1–C) (Chen, C. and Chen, 2002; Eulgem et al., 2000).

WRKY TFs have been shown to play both positive and negative roles during the regulation of plant defence responses (Pandey and Somssich, 2009). Regulation of WRKY activity by MAPKs plays a role not only in substrate phosphorylation, but also in the sequestration and release of TFs, which allows access to target promoters. On the other hand, activation of WRKY TFs by MAPKs can be caused by phosphorylation-induced structural changes (Ishihama and Yoshioka, 2012).

Furthermore, WRKYs also play a role in a complex hormone signaling network. They are involved in the antagonistic functions of jasmonic acid (JA)/ethylene (ET) and salicylic acid (SA), function up– and downstream of hormone signals and regulate developmental processes via auxins, brassinosteroids and cytokinins (Agarwal et al., 2011; Antoni et al., 2011; Guo and Gan, 2005; Nilsson et al., 2010; Rushton et al., 2012).

In addition, WRKYs actively participate in the control of seed, embryo, microphyle, and endosperm development (Jiang et al., 2013; Kang et al., 2013; Li, J. et al., 2013; Ngo et al., 2012).

1.3 Micoble associated molecular partern (MAMP)

The plant immune system is comprised of surveillance systems that perceive several general microbe elicitors, which allow plants to switch from growth and development into a defense mode. The elicitors are essential structures for the microbes and are conserved among pathogens. These elicitors are called microbe- or pathogen-associated molecular pattern (MAMP or PAMP). MAMPs or PAMPs are recognized by pattern recognition

receptors (PRRs) of the plant innate immune system, which are localized on the surface of plant cells (Ausubel, 2005; Jones and Dangl, 2006).

Table 1 summarizes known microbe-associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs) and their respective pattern recognition receptors (PRRs) in plants. Some of PRRs have not been yet identified.

Examples for MAMPs are flg22 and elf18 derived from the bacterial flagellin (Flg) and the translation elongation factor Tu (EF-Tu), respectively. They have been extensively studied for MTI in plants (Felix and Boller, 2003; Zipfel et al., 2006).

The MAMP peptidoglycan (PGN) supplys rigidity and structure to the cell envelopes of both Gram-negative and Gram-positive bacteria (Erbs et al., 2008; Willmann et al., 2011). This and lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria have been found to act as MAMPs of plant innate immunity (Erbs and Newman, 2012; Silipo et al., 2005).

Oligosaccharides derived from cell wall polymers of fungi and oomycetes also act as MAMPs. Fungal chitin and its degraded N-acetyl-chito-oligosaccharides products, chitin oligomers induce diverse defense responses in both monocot and dicot plants (Kaku et al., 2006; Miya et al., 2007).

DAMPs are molecules released by wounded cells and act as endogenous danger signals to promote and exacerbate the defense response (Boller and Felix, 2009; Lotze et al., 2007; Seong and Matzinger, 2004). For instance, the cytosolic systemin is released upon cell damage and acts as DAMP on neighbouring cells (Narvaez-Vasquez and Ryan, 2004). Similarly, oligogalacturonides (OG) and cutin are secreted from plant cell walls in injury cases (Denoux et al., 2008; Schweizer et al., 1996). AtPep1 is a 23 aa- peptide that activates genes in pathogen attacks (Huffaker et al., 2006) and will be described in more detail in the next chapter.

Table 1. Microbe-associated molecular patterns (MAMPs) and Damage-associated molecular patterns (DAMPs).

Name	**Pattern recognition receptors (PRRs)**	**References**
MAMPs		
Flagellin (Flg; flg22)	FLS2 (*Arabidopsis*)	(Felix et al., 1999; Gómez-Gómez and Boller, 2000)

Pep25 from a surface Glycoprotein of *Phytopthora Sojae*)	Not identified	(Nürnberger et al., 1994; Rushton et al., 1996)
Elongation factor TU (EF-Tu; elf18/26)	EFR (Arabidopsis; Brassicaceae)	(Kunze et al., 2004)
Peptidoglycan (PGN)	Lym1 and Lym3 (*Arabidopsis*)	(Erbs et al., 2008; Gust et al., 2007; Willmann et al., 2011)
Lipopolysaccharide (LPS)	Not identified	(Newman, M. et al., 1995)
Bacterial cold shock proteins (RNP1 motif)	Not identified	(Felix and Boller, 2003)
Bacterial superoxide dismutase (Sod)	Not identified	(Watt et al., 2006)
Activator of XA21 (Ax21)	XA21 and XA21D (rice)	(Lee et al., 2009; Song et al., 1995; Wang et al., 1998)
Beta-Glycan (GE)	GEBP (putative receptor soyabean)	(Darvill and Albersheim, 1984; Umemoto et al., 1997)
Chitin	CeBip and CERK1 (rice); AtCERK1 (*Arabidopsis*)	(Felix et al., 1993; Kaku et al., 2006; Miya et al., 2007; Shimizu et al., 2010)
Avirulence on Ve1 tomato (Ave1)	Ve1 (putative tomato receptor)	(de Jonge et al., 2011; Kawchuk et al., 2001; Thomma et al., 2011)
Xylanase (EIX)	EIX (tomato)	(Bailey et al., 1990; Ron and Avni, 2004)
Pep-13 (An oligopeptide of 13 amino acids From *P. mega-sperma*)	Not identified	(Nürnberger et al., 1994)
Cellulose-binding elicitor lectin (CBEL) from Phytophthora	Not identified	(Gaulin et al., 2006; Mateos et al., 1997; Séjalon-Delmas et al., 1997)
DAMPs		
Systemin	Not identified	(Narvaez-Vasquez and Ryan, 2004)
AtPep1 (23 aa part of a cytosolic protein from *Arabidopsis*)	PEPR1 (*Arabidopsis*)	(Huffaker et al., 2006; Yamaguchi et al., 2006)
Oligogalacturonides (OGs)	WAK1 (*Arabidopsis*)	(Brutus et al., 2010; Nothnagel et al., 1983)
Cutin	Not identified	(Kauss et al., 1999; Schweizer et al., 1996)

(Modified from Newman, M. A. et al., 2013)

1.3.1 MAMPs and DAMPs used in this study

Bacterial MAMPs

Flagellin 22 (flg22)

Flg22 is a 22 amino acid peptide, a highly conserved domain in the N-terminal part of the bacterial Flagellin (Felix et al., 1999). Flg22 interacts with a specific PRR (FLS2) that harbours both an extracellular LRR domain and an intracellular kinase domain in *Arabidopsis* (Chinchilla et al., 2006; Gómez-Gómez et al., 2001). The recognized domain within Flagellin (Flg) is different in all plant species. For example flg15 was shown to be highly active in tomato, while it only leads to immune responses at higher concentration in *Arabidopsis*. Rice can recognize flg22, but its defense responses is greater to the full length Flg (Takai et al., 2008). The recognition of the flg epitope is not limited to different plant families. The flg15, a 15 aa peptide derived from *E. coli* was shown only to be highly active in tomato (*Solanum lycopersicum*) but not in tobacco. Furthermore, the SlFLS2, an ortholog of the *Arabidopsis* FLS2 receptor, is the Flg perception system specific for tomato (Robatzek et al., 2007).

Flg perception by FLS2 involves a complex formation with the Brassinosteroid-Insensitive 1 (BRI1)-associated receptor kinase 1 (BAK1) in *Arabidopsis* (Chinchilla et al., 2007). BAK1 is required for the immune responses triggered by multiple MAMPs (Roux et al., 2011). The activities of MAP kinases (MAPK) were delayed and reduced or even absent in response to flg22 in bak1 mutants compared to wild type plants. This result has shown that BAK1 acts as a positive regulator of signaling in *Arabidopsis*. The FLS2 interaction with BAK1 in a ligand dependent manner by flg22 allows phosphorylation and activation of the receptor complex (Schulze et al., 2010; Chinchilla et al., 2007). After dimerization of the FLS2-BAK1 receptor complex, Botrytis-induced kinase 1 (BIK1) associate with FLS2, allowing BIK1 to phosphorylate downstream components of the FLS2-BAK1 complex, and thus linking the MAMP receptor complex to downstream intracellular signaling, resulting in activation of MTI (Lu et al., 2010).

Fungal MAMPs

Pep25

Pep25, an oligopeptide of 25 amino acids, is derived from a surface glycoprotein of *Phytophthora sojae*. It was shown to be necessary and sufficient to stimulate a complex

defense response in parsley cells. Pep25 functions as a MAMP in the parsley protoplast system (Nürnberger et al., 1994); (Rushton et al., 1996)

DAMPs

AtPep1, a 23-aa peptide from *Arabidopsis*, is an endogenous peptide that activates genes specifically for defense against pathogens (Huffaker et al., 2006). The AtPep1 receptor, PEPR1 (PEP receptor 1), like FLS2 and EFR, belongs to LRR XI subfamily of LRR receptor kinases (Yamaguchi et al., 2006). Based on sequence similarity of PEPR1 a second receptor, PEPR2, has been identified. Transcription of PEPR1 and PEPR2 is activated by wounding, Methyl-Jasmonate (MeJA), peptides, and specific MAMPs (Yamaguchi et al., 2010).

1.4 Synthetic promoters

The promoters of pathogen induced genes contain *cis*-elements that can cause an inducible expression independent of the native promoter context (Rushton et al., 2002). These *cis*-elements may be used for the production of synthetic promoters, such as shown in Figure 2. It has already been shown that native pathogen-induced promoters, unlike synthetic promoters are less suitable (Kooshki et al., 2003). The strength and inducibility of artificial promoters is related to the number, sequence, and combination of different *cis*-elements which can be freely selected. Also, the distance to each other or to the TATA box may have a strong influence on the function of the *cis*-elements (Rushton et al., 2002). Synthetic promoters have been successfully used in studies in plants, either to examine the role of *cis*-elements or to regulate the inducibility of the target gene (Pua and Davey, 2010). There have already been studies of *cis*-elements in connection with heat shock (Pietrzak et al., 1989), development (Puente et al., 1996), light (Gilmartin et al., 1990), tissue-specificity (Ni et al., 1996), cold stress (Zhu et al., 2008) or wounding and pathogen infestation (Rushton et al., 2002). In those studies, synthetic promoters were versatile in use. They can also be used as a reporter for mutant screens, such as in the study of (Oono et al., 1998).

Pesticides are usually used for the control of pathogens (Moffat, 2001). An alternative to the conventional methods of pathogen control using pesticides is the use of synthetic promoters in plants. Examples for improvements of plant defenses by synthetic promoters are interference with the replication of viruses in the plant, the expression of gene

products toxic to pathogens or the improvement of plant in resistance mechanisms (Rushton et al., 2002). In addition, it is also possible to use transgenic plants which contain synthetic promoters habouring resistance (*R*) genes as an early warning system in infected plants (Mazarei et al., 2008).

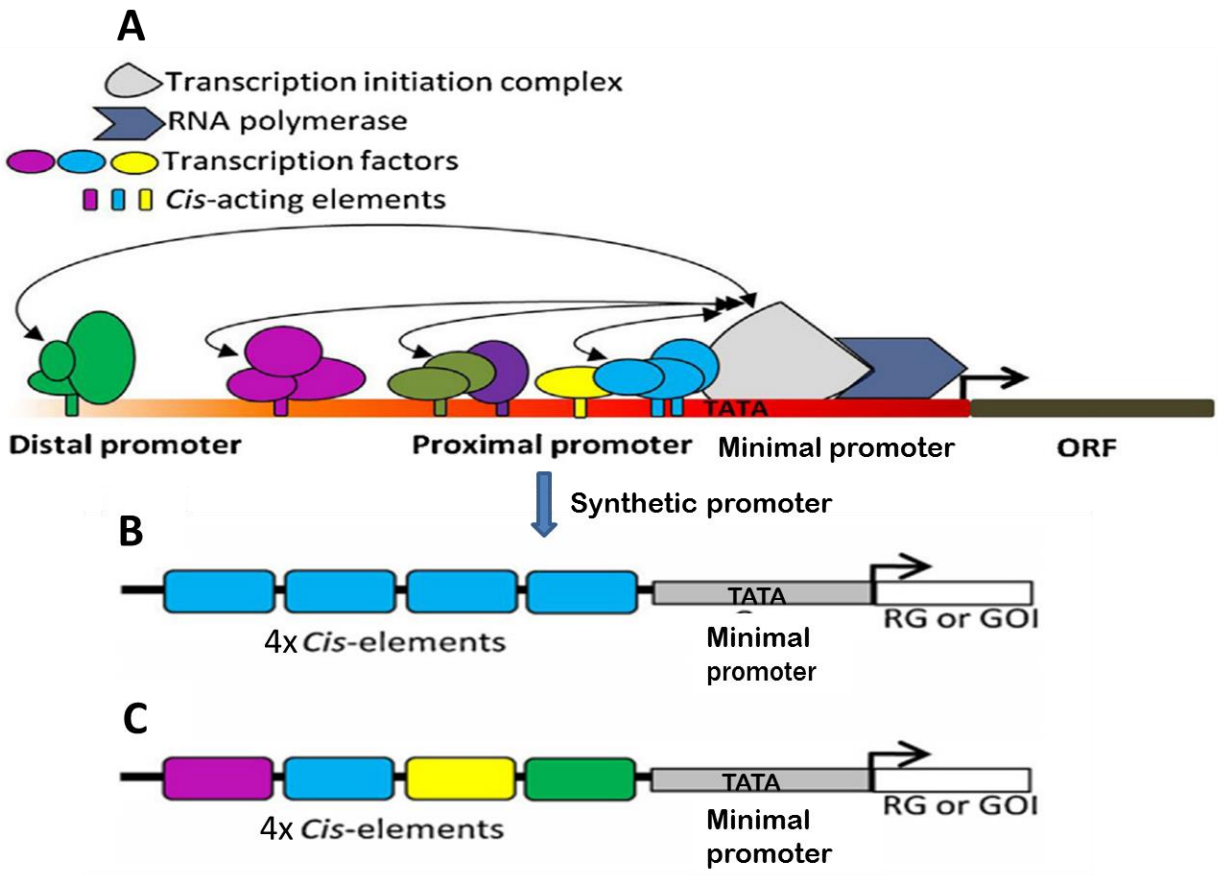


Figure 2. Design of a synthetic promoter

Representative structure of synthetic promoters. (A) Basic model of transcriptional regulation of protein-encoding genes. (B) Tetrameric repeats of the same regulatory element driving either a reporter gene (RG) or other gene of interest (GOI). (C) Example of a synthetic promoter-containing different regulatory elements controlling a reporter gene (RG) or a gene of interest (GOI). (Modified from Hernandez-Garcia and Finer, 2014).

A further possibility to modify the plant immune system is to express cell death-inducing genes in an infection in order to counteract pathogen infection of the plant (Niemeyer et al., 2014). For this purpose, well-regulated *cis*-elements with low background activity must be used for a synthetic promoter. Such ideal *cis*-elements have not been identified until

now. Our aim is to identify new pathogen-responsive *cis*-elements in order to expand the range of synthetic promoter design.

1.5 Identification of novel *cis*-elements by combination of bioinformatic and experimental analysis

For the identification of unknown *cis*-elements there are different approaches. Koschmann *et al*. have found an effective way of combining bioinformatic and experimental analysis of *cis*-elements which originate from promoters of genes that are coregulated during pathogen response (Koschmann et al., 2012). The strength of the study is that they cover a wide range of pathogens or MAMPs. Here, the focus of the investigation was mainly on fungal pathogens and stimuli. The database PathoPlant (Bülow et al., 2007), that contains microarray data from *A. thaliana,* served as starting material for the bioinformatic analysis. In Figure 3A the main steps of the procedure are shown.

510 MAMP upregulated gene groups were identified. This was done using a database query with the combination of up to six different stimuli. The promoters (1000 bp upstream to the transcription start) of the gene groups were analyzed by the software BEST (Binding Site Estimation Suite of Tools, Che et al., 2005) for conserved *cis*-regulatory elements. This was done under the assumption that genes are regulated by the same transcription factors. As a result of this study, a total of 407 different sequence motifs were identified. In the next step these were classified into 37 groups of motifs using STAMP, and STAMP was also used for further investigation (Mahony and Benos, 2007). STAMP is a web tool, which can detect similarities of DNA-binding motifs. From the 37 groups a relationship tree was subsequently created, which is shown in Figure 3 B. Then similarities were searched between the 37 groups and known *cis*-elements. For this purpose STAMP compared the motifs of the 37 groups to the already known *cis*-sequences from the databases AthaMap, AGRIS and PLACE (Bülow et al., 2006; Higo et al., 1999; Palaniswamy et al., 2006). In addition to the numerous unknown transcription factor binding sites (TFBS) some well-known TFBS were represented, which are involved in the plant immune system such as TF families MYB, AP2/ERF and WRKY (Tsuda and Somssich, 2015).

After the bioinformatic search, the experimental analysis of *cis*-elements was done with synthetic promoters. These promoters consist of tetramers of individual *cis*-sequences,

which are connected by linker sequences. The investigations were carried out in the parsley protoplast

system, in which the MAMP from *Phytophthora sojae* Pep25 acts as elicitor. This way, 25 elicitor-responsive sequences were identified. A detailed analysis defined a novel core nucleotide stretch, GACTTTT, that is present in almost all of the seven Pep25-responsive sequences in motif group 27 (Koschmann et al., 2012). Notably, only three of them (seq21, 22 and 24) contain the classical W-box, TTGACY (Y=C/T), the binding site for WRKYs while the others do not. The absence of the W-box or even its core sequence TGAC in the remaining sequences (seq18, 19, 20 and 23) suggested the contribution of the novel identified GACTTTT sequence in pathogen response. A subsequent study by Machens *et al*. demonstrated that the transcription factor (TF) WRKY70 binds directly to the sequence YGACTTTT (Y=C/T) in sequence 20 and that this sequence is significantly enriched in WRKY70-upregulated genes and is required for WRKY70-activated reporter gene expression in *Arabidopsis* (Machens et al., 2014). In contrast, the similar sequences GGACTTTT and GGACTTTG that harbour the core sequence GACTTT of the designated WT-box (Bolivar et al., 2014; Machens et al., 2014) were not found to interact with any TFs in yeast one-hybrid screenings although they are important in MAMP responsivity (Lehmeyer et al., 2016).

The above studies highlight the diversity as well as the differential functions of these newly-identified WT-boxes in gene regulation during pathogen attacks. They provide additional information to the well-known W-boxes which are essential for WRKY interaction. Interestingly, both, sequence 22 and 24, now designated *cis*-regulatory module (CRM) 1 and CRM2 respectively, are present in the promoter of *AtWRKY30* (At5g24110) (Figure 4). They contain two W-boxes with the core sequence TGAC and one WT-box with the above described core sequence. In case of CRM2, the WT-box overlaps with its adjacent W-boxes. *WRKY30* is upregulated by reactive oxygen species (Scarpeci et al., 2008). In addition, *Arabidopsis* plants overexpressing *WRKY30* are more tolerant than wild-type plants to oxidative and salinity stress during seed gemination (Scarpeci et al., 2013). WRKY70 and WRKY30 interact in a yeast two-hybrid screen and are involved in developmental leaf senescence (Besseau et al., 2012). In addition, CRM1 and CRM2 of *WRKY30* were shown to bind to WRKY70 in gel shift assay (Machens et al., 2014),

indicating the participation of either single or double W- and/or WT-box of these CRMs in transcriptional reprograming.

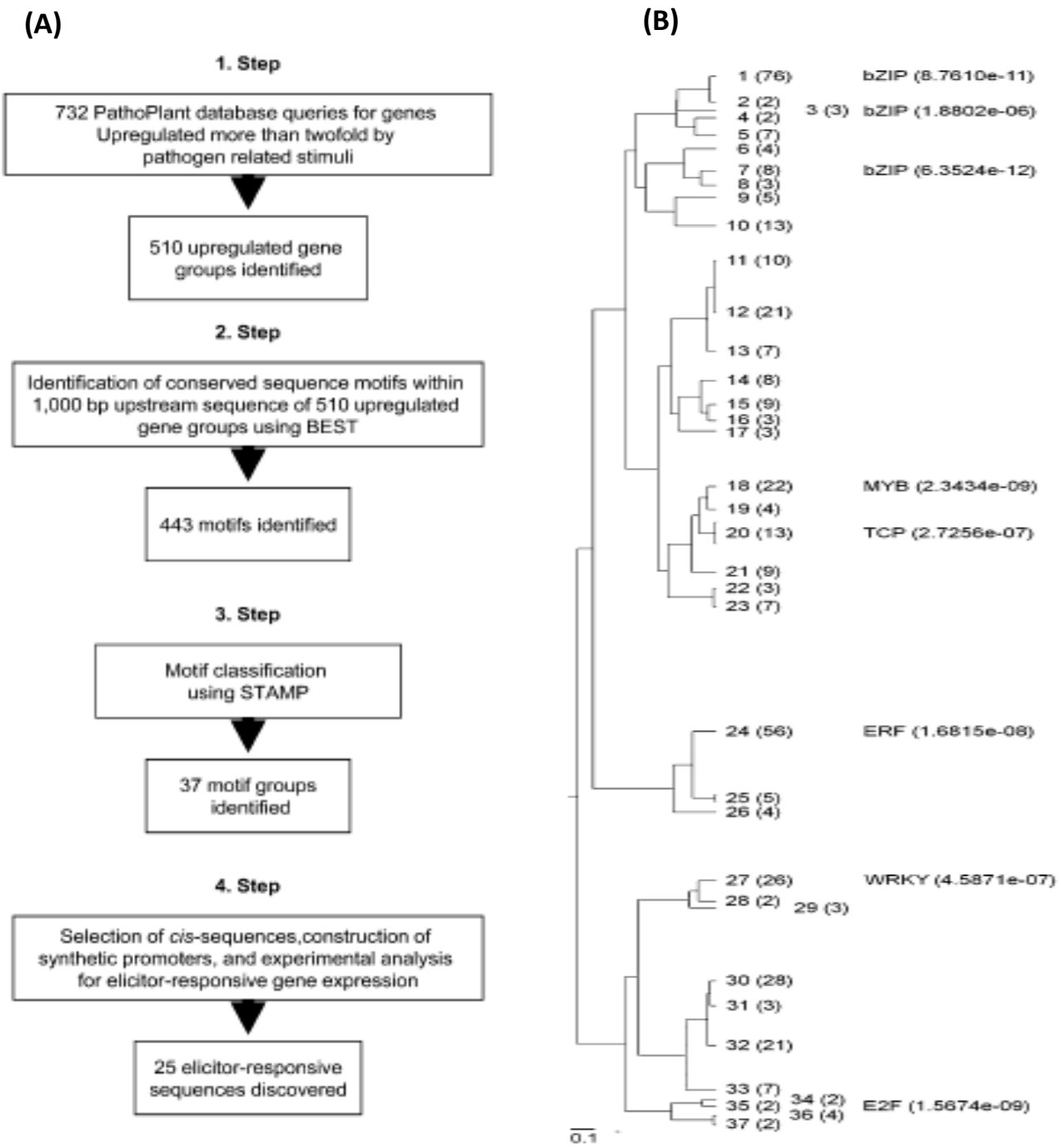


Figure 3. Bioinformatics and experimental operations to identify pathogen responsive *cis*-sequences

(A) Schematic representation of the bioinformatic and experimental analysis of pathogen-responsive, conserved sequence motifs (B) Relationsship tree of the identified sequence motifs based on their similarities. The number of motifs in each of the 37 motif families is given in parentheses. Motif groups showing high similarity to known *cis*-regulatory sequences are indicated by the designation of the corresponding TF family (Koschmann et al., 2012).

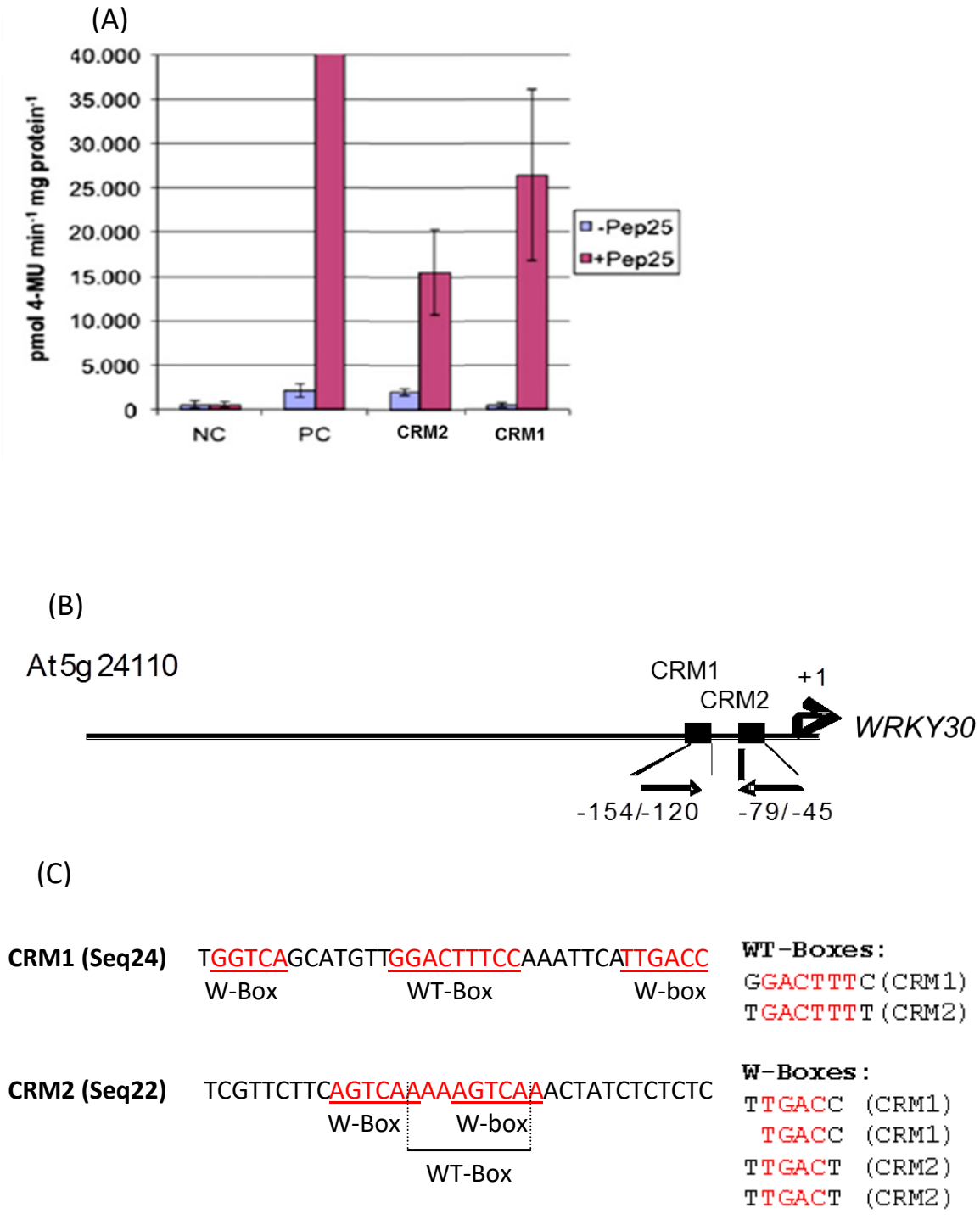


Figure 4. The WRKY30 promoter with the two *cis*-regulatory modules (CRMs).

(A) Pep25-responsive *cis*-sequences, Seq24 (CRM1) and Seq22 (CRM2). (B and C) CRM1 and CRM2 each harbouring two W- and one WT-box (Modified from Koschmann et al., 2012; Machens et al., 2014).

2. Objectives

The aim of this study was to gain more insight into the regulation of the two cis-regulatory modules CRM1 and CRM2 from the *WRKY30* promoter and to understand the role of the W- and WT-boxes for MAMP-responsive gene expression.

The participations of CRM1 and CRM2 in the *WRKY30* promoter for MAMP responsivity should be studied in detail to answer the following questions:

1. Which boxes in the native *WRKY30* promoter are required for Pep25-responsive gene expression in parsley protoplasts?
2. Which boxes in CRM1 are required for Pep25-responsive gene expression in parsley protoplasts?
3. Are CRM1 and CRM2 required for flg22- and AtPep1-responsive gene expression in *Arabidopsis* protoplasts?
4. Which boxes in CRM1 are required for flg22- and AtPep1-responsive gene expression in *Arabidopsis* protoplasts?
5. Which transcription factors (TFs) interact with CRM1 and CRM2?
6. How do these TFs regulate reporter gene expression by CRM1?

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals and enzymes

Standard chemicals from PanReac AppliChem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Sigma-Aldrich (Munich, Germany) companies were used. PEG-4000 from Sigma-Aldrich (Munich, Germany) was used (item no. 81242) for the transformation of *Arabidopsis* protoplasts, as this is critical for high transformation efficiency (Yoo et al., 2007).

Common restriction enzymes and DNA modifying enzymes (such as phosphatase, T4 ligase, etc.) were purchased from Thermo Fisher Scientific (Rockford, IL, USA).

3.1.2 Agarose gel electrophoresis

50X TAE

2 M TRIS

1 M Acetic acid

0.05 M EDTA, 0.5 M stock solution, pH 8.0

10x DNA loading buffer

0.25 % (w/v) bromophenol blue

0.25 % (w/v) xylene cyanol

25 % (v/v) Ficoll 400

3.1.3 Antibiotic and herbicide solutions

All antibiotics are dissolved in dH_2O, sterilized by filtration by a syringe filter with a pore size 0.45 µm, then stored at -20 °C.

Carbenicillin stock solution

100 mg/ml carbenicillin disodium salt

The working concentration for selection of bacteria is 50 -100 mg/l.

Kanamycin stock solution

100 mg/ml kanamycin sulfate

The working concentration for the selection of bacteria is 50 mg/l.

3-amino-1,2,4-triazoles (3-AT) stock solution

1 M 3-Amino-1,2,4-triazoles

Dissolve, sterilize by filtration and store for one month at 4 °C in dH_2O.

The working concentration in Y1H screening is 50 -100 mM.

3.1.4 Plasmid Preparation from *E. coli*

Silica suspension

10 % (w/v) silicon dioxide (Sigma-Aldrich, Munich, Germany)

Storage at 4 °C, before use thoroughly resuspend.

Solution A (Resuspension solution)

50 mM Tris-HCl, from 1 M stock solution, pH 7.5

10 mM EDTA

100 mg/l RNase A

Storage at 4 °C.

Solution B (Alkaline lysis solution)

0.2 M NaOH

0.1 % (w/v) SDS

Solution C (Neutralization solution)

1.32 M potassium acetate

Adjust with acetic acid pH 4.8.

Solution D (NaI solution)

6 M NaI

Storage at 4 °C in the dark.

Solution E (Washing solution)

50 % (v/v) ethanol

10 mM Tris-HCl 1 M stock solution pH 7.5

100 mM NaCl

1 mM EDTA

TE

10 mM Tris-HCl, 1 M stock solution, pH 8.0

1 mM EDTA, 0.5M stock solution, pH 8.0

LTE

10 mM Tris-HCl, 1 M stock solution, pH 7.5

0.1 mM EDTA, 0.5 M stock solution, pH 8.0

3.1.5 Plasmid preparation from yeast cells

Plasmid preparation from yeast cells

Cell suspension buffer (A)

50 mM glucose

10 mM EDTA

25 mM Tris-HCl, pH 8.0

150 U/ml Lyticase (Sigma-Aldrich, Munich, Germany)

Store aliquots at -20 ° C.

Denaturation solution (B)

0.2 M sodium hydroxide

1% SDS

Renaturation solution (C)

3 M potassium acetate

Adjust with acetic acid pH 4.8.

3.1.6 Growing *A. thaliana*

Sterilizing solution

3 % (v/v) sodium hypochlorite

0.1 % (v/v) Triton X-100

Add sterile distilled H_2O, filtered sterilize, store at 4 °C.

0.1 % agarose

0.1 % (w/v) agarose

Add dH_2O and autoclave.

3.1.7 Transformation of yeast cells

Lithium solution

1 M lithium acetate dihydrate

Prepare and sterilize by filtration.

50 % PEG 3350

50 % (w/v) PEG 3350 (Sigma-Aldrich, Munich, Germany)

Carrier DNA

2 mg/ml salmon sperm DNA (Sigma-Aldrich, Munich, Germany)

Dissolve at 4 °C in sterile TE, aliquot and store at -20 °C

For denaturing: 5 min heating in boiling water, then cool immediately in ice water and store until use.

3.1.8 Isolation and transformation of Parsley and *Arabidopsis* protoplasts

Parsley

$CaCl_2$ solution

0.24 M $CaCl_2$

$Ca(NO_3)_2$-MES solution

0.275 M $Ca(NO_3)_2$

2 mM MES, 100 mM stock solution

Adjust with KOH pH 6.0.

Enzyme solution

0.5 % (w/v) Cellulase R-10 Onozuka (Duchefa, Haarlem, Netherlands)

1.08 % (w/v) Macerozyme R-10 (Duchefa, Haarlem, Netherlands)

0.24 M $CaCl_2$

Prepare fresh, stir for 2 h and sterilize by filtration.

PEG solution

25 % (w/v) PEG 6000

100 mM $Ca(NO_3)_2$

45 mM mannitol

Pep25 solution

100 µg/ml Pep25 peptide

Prepare on ice. Adjust with KOH to pH 9.0, sterilize by filtration and store aliquots at -20 °C.

P5 medium

1X Gamborg B5 medium (Duchefa, Haarlem, The Netherlands)

add

1 mg/l 2,4-dichlorophenoxyacetic acid (10 ml) from 0.01 % (w/v) stock solution

0.28 M sucrose (96.9 g)

Adjust with 1 M KOH pH 5.7, autoclave and store in dark at RT.

Arabidopsis

Enzyme solution

1% (w/v) Cellulase Onozukara R-10 (Duchefa, Haarlem, Niederlande)

0.25% (w/v) Macerozym R-10 (Duchefa, Haarlem, Niederlande)

20 mM KCl (1 M stock solution)

20 mM MES (0.2 M stock solution), pH5.7

Prepare fresh, incubate enzymes at 55 °C for 10 min to dissolve and cool to room temperature

Addition

10 mM $CaCl_2$ (1 M stock solution)

0.1 % BSA (10 % stock solution)

filter before use through a filter with 0.45 µm pore size

W5

154 mM NaCl (1 M stock solution)

125 mM CaCl2 (1 M stock solution)

5 mM KCl (2 M stock solution)

2 mM MES (0.2 M stock solution), pH 5.7

Prepare fresh

MMg

0.4 M Mannitol (1 M stock solution)

15 mM $MgCl_2$ (2 M stock solution)

4 mM MES (0.2 M stock solution), pH5.7

Store at room temperature

30 % PEG-Solution

30 % (w/v) PEG 4000 (Sigma-Aldrich, München, Deutschland)

0.1 M CaCl2 (1 M stock solution)

0.2 M Mannitol (0.8 M stock solution)

prepare fresh and keep up to three days at room temperature

B5 Medium (1 L)

1x Gamborg B5 Medium (Duchefa, Haarlem, Niederlande)

Added

79.27 g Glucose-Monohydrat

30 g Saccharose

1000 µl 2,4-D (0.01% (w/v) stock solution)

150 µl 6-BAP (0.01% (w/v) stock solution)

Adjust to pH 5.8 with 1M KOH, sterilize by filtration, store at -20 °C

3.1.9 Protein determination according to Bradford

Bradford working solution

2 ml 5x Roti®-Quant reagent (Carl Roth, Karlsruhe, Germany)

5.5 ml dH2O

Use freshly prepared solution.

3.1.10 Luciferase assays

Luciferin stock solution

10 mM D-luciferase

25 mM glycylglycine, pH 7.8

Store at -20 °C in aliquots.

LUC extraction buffer

0.1 M NaH_2PO_4, pH 7.8

1 mM dithiothreitol (DTT) from 1 M stock solution (store at -20 °C) fresh DTT.

LUC reaction buffer

15 mM $MgSO_4$

25 mM glycylglycine, pH7.8

Store at -20 °C.

Immediately before use, add 5 mM ATP, 100 mM stock solution (stored at -20 °C).

LUC-substrate solution

0.2 mM stock solution of luciferin

25 mM glycylglycine, pH 7.8

Prepare immediately before use.

3.1.11 β-glucuronidase assays

GUS extraction buffer

50 mM $NaPO_4$, pH 7.0

10 mM Na_2EDTA

0.1 % (v/v) Triton X-100

0.1 % (v/v) N-laurylsarcosine

10 mM β-mercaptoethanol

add fresh β-mercaptoethanol before use, store at 4 °C.

GUS reaction buffer

GUS extraction buffer added:

1 mM 4-MUG (4-methylumbelliferyl-β-D-glucuronide dihydrate)

Use freshly prepared solution.

3.1.12 Gelshift-Assays

10x Gelshift-Binding buffer

100 mM TRIS

500 mM KCl

10 mM Dithiothreitol (DTT)

Add fresh DTT before use, pH 7.5

5x TBE

450 mM TRIS

450 mM Borsäure

10 mM EDTA

pH 8.3

3.1.13 Media

All media were prepared with dH_2O, autoclaved for 20 min at 121 ^{o}C and stored at RT. After autoclaving, medium is cooled to 50 °C if necessary sterile antibiotics, glucose solution or 3-AT were added.

LB medium (1000 mL)

10 g Bacto Tryptone

5 g yeast extract

10 g NaCl

15 g agar (optional)

dYT medium (1000 ml)

16 g Bacto Tryptone

10 g yeast extract

5 g NaCl

15 g agar (optional)

SOC medium (1000 ml)

20 g Bacto Tryptone

5 g yeast extract

584 mg NaCl

186 mg KCl

2.033 g $MgCl_2.6(H_2O)$

2.465 g $MgSO_4.7(H_2O)$

Adjust to pH 6.8 to 7.0. Add 10 ml of 2 M glucose solution (filter sterilized), make aliquots and store at -20 °C.

10x amino acids mix (drop-out mix) (1000 ml)

For use in drop-out media for selection of plasmids in yeast, the desired amino acids are left out of the mix below.

200 mg L-adenine hemisulfate

200 mg L-arginine HCl

200 mg L-histidine HCl monohydrate

300 mg L-isoleucine

1,000 mg L-leucine

300 mg L-lysine HCl

200 mg L-methionine

500 mg L-phenylalanine

2.000 mg L-threonine

200 mg L-tryptophan

300 mg L-tyrosine

200 mg L-uracil

1,500 mg L-valine

Store at 4 °C.

SD medium (1000 ml)

6.7 g yeast nitrogen base without amino acids (BD, Franklin Lakes, United States)

100 ml 10x amino acids mix (or drop-out mix for selection)

18 g agar (optional)

Add to 950 ml with dH_2O and adjust pH 5.6 with NaOH.

Autoclave and add 50 ml of 40 % glucose solution (filter sterilized)

2x YPAD medium (1000 ml)

20 g yeast extract

40 g peptone

80 mg L-adenine hemisulfate

18 g agar (optional)

Add 900 ml dH_2O; autoclave, then add 100 ml of 40 % glucose solution (filter sterilized).

YPAD medium (1000 ml)

10 g yeast extract

20 g peptone

80 mg L-adenine hemisulfate

18 g agar (optional)

Fill with dH_2O to 950 ml and autoclave. Then add 50 ml of 40 % glucose solution (filter sterilized)

Fe-EDTA solution (500 ml)

2.780 mg $FeSO_4.7H_2O$

3.730 mg Na_2EDTA

Add 500 ml dH_2O, store at 4 °C.

2,4-D (2,4-dichlorophenoxyacetic acid) solution (500 ml)

50 mg 2,4-D

The 2,4-D is dissolved in 5 ml 100 % ethanol then added 495 ml dH_2O. Store at 4 °C.

Trace elements (100 ml)

300 mg H_3BO_3

1.120 mg $MnSO_4.H_2O$

300 mg $ZnSO_4.7H_2O$

25 mg $Na_2MoO_4.2H_2O$

39 mg $CuSO_4.5H_2O$

25 mg $CoCl_2.6H_2O$

Add 100 ml ddH_2O, sterilize by filtration and store as aliquots.

B5 vitamins (500 ml)

5,000 mg Myo-inositol

50 mg Nicotinic acid

50 mg Pyridoxine HCl

500 mg Thiamine HCl

Add 500 ml dH_2O, sterilize by filtration and stored as aliquots at -20 °C.

HA medium (1000 ml)

20 ml KNO_3 from 1.236 M stock solution

1.14 ml $CaCl_2.2H_2O$ from 0.582 M stock solution

2 ml $MgSO_4.7H_2O$ from 0.507 M stock solution

2 ml $(NH_4)_2SO_4$ from 0.507 M stock solution

2 ml $NaH_2PO_4.H_2O$ from 0.543 M stock solution

1 ml of 2,25 mM stock solution KI

2.5 ml Fe-EDTA from Fe-EDTA solution

1 ml trace elements from stock solution

10 ml B5 vitamins from stock solution

10 ml 2,4-D from a stock solution

20 g sucrose

Add ddH_2O to about 700 ml, adjust pH 5.5 with KOH/HCl. Fill up to 1000 ml with ddH_2O. In 40 ml portions in 200 ml Erlenmeyer flasks, autoclave (20 min, 121 °C). Immediately after sterilization from the autoclave, cool to room temperature and store in the dark.

3.1.14 Bacterial and yeast strains

Agrobacterium tumefaciens

For transformation purposes the nononcogenic *A. tumefaciens* strain C58C1 (Deblaere *et al.,* 1985), which has the disarmed Ti plasmid pGV2260, was used. Carbenicillin (50 mg/l) and Rifampicin (50 mg/l) were used for selection.

Escherichia coli

For cloning, the *E. coli* strains XL1 Blue MRF' (Agilent Technologies, Böblingen, Germany), INVαF' and TOP10 (both Life Technologies, Darmstadt, Germany) were used.

Saccharomyces cerevisiae

For the Y1H screening, *S. cerevisiae* strain Y1HGold (Clontech, Saint-Germain-en-Laye, France) was used.

3.1.15 Plant material

Arabidopsis thaliana

Arabidopsis thaliana ecotype Columbia (Col-0) was used. Seeds of this ecotype were already in the laboratory (Prof. Dr. Reinhard Hehl).

Petroselinum crispum cell culture

For the isolation and transformation of parsley protoplasts, a suspension culture of *Petroselinum crispum* Pc 5/3 was used (provided by courtesy of Dr. Imre Somssich, Max Planck Institute for Plant Breeding Research, Köln).

3.1.16 Oligonucleotides and peptides

Primers and oligonucleotides for PCR applications or cloning were synthesized at Life Technologies (Darmstadt, Germany) or Eurofins MWG Operon (Ebersberg, Germany). They were dissolved to a concentration of 100 pmol/µl in LTE buffer. For PCR, the primers are each diluted 1: 5 with ddH_2O and stored at -20 °C.

All oligonucleotides and primers used in this work are listed in the Appendix, Table 12.

The peptide elicitors flg22 (QRLSTGSRINSAKDDAAGLQIA) and AtPep1 (ATKVKAKQRGKEKVSSGRPGQHN) were synthesized by PANATecs GmbH (Heilbronn, Germany).

The Pep25 peptide with the amino acid sequence DVTAGAEVWNQPVRGFKVYEQTEMT has been synthesized at SeqLab (Göttingen, Germany).

3.1.17 Vectors

The following plasmids were used in this work. The associated lab numbers can be found in the Appendix, Table 13, Vectors.

pBT10GUS-d35SLUC: Derivative of pBT10GUS carrying a constitutively expressed LUC gene (Koschmann et al., 2012).

pBT10GUS-d35SLUC_D: Vector is modified from pBT10GUS-d35SLUC without TATA box of the GUS gene.

pBT10LUC: Vector for transient expression assays in *Arabidopsis protoplasts* (Sprenger-Haussels and Weisshaar, 2000).

pCR2.1: Cloning (Life Technologies, Darmstadt, Germany).

pRT103GUS: Vector constitutively expressing the *uid*A reporter gene (Töpfer et al., 1987). It is used as a transformation control for *Arabidopsis protoplasts.*

pHis2.1: Bait vector for yeast one hybrid screening (Clontech, Saint-Germain-en-Laye, France).

pORE-O2-d35S-pA: T-DNA vector with a kanamycin resistance gene for the expression of the transcription factors in protoplasts, or for overexpressing in plants. Derivative of the T-DNA vector pORE-O2 (Coutu et al., 2007), modified by (Machens et al., 2014).

3.1.18 Kits

For experiments with DNA (cloning, purification, plasmid isolation), the following kits were used:

- EasyPrep Pro Plasmid Miniprep Kit (Biozym, Hessisch Oldendorf, Germany)
- NucleoBond Xtra Midi EF (Macherey-Nagel, Düren, Germany)
- NucleoBond Xtra Maxi EF (Macherey-Nagel, Düren, Germany)
- NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany)
- NucleoSpin Plant II (Macherey-Nagel, Düren, Germany)
- RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Rockford, IL, USA)
- TA Cloning Kit with pCR2.1 vector (Life Technologies, Darmstadt, Germany).

3.1.19 Other materials

Plastic materials used in this study (such as petri dishes, tips, Eppendorf tubes, Falcon tubes, cuvettes, Pasteur pipettes) have been ordered from Sarstedt AG & Co company (Nümbrecht, Germany).

3.1.20 Equipment

In addition to the common laboratory centrifuges, incubators, vortex and sterile benches, the following devices were used:

- Gene Pulser II Electroporation, Bio-Rad Laboratories GmbH
- Gel Doc XR system, Bio-Rad Laboratories, Inc
- Gel electrophoresis, Biozym
- Growth chamber, CU-32L and CU-36L/4, Percival Scientific
- Microplate Reader, TriStar LB 941, Berthold Technologies
- NanoPhotometer™, Implen GmbH
- PCR thermocycler, Mastercycler® personal, Eppendorf AG
- Photometer, Ultrospec 2000 UV/VIS Spectrophotometer, Pharmacia Biotech
- High Vacuum Pump E1M5, Edwards
- Hemocytometer Cell Counting Chamber, Hemacytometer, Merck KGaA, Darmstadt, Germany
- Heraeus Biofuge Fresco (Heraeus, Germany)

- Thermo Scientific Sorvall Evolution RC centrifuge
- Beckman Coulter Avanti® J-26 XP
- Beckman GS-6KR centrifuge

3.2 Methods

3.2.1 DNA constructs

If not otherwise specified, standard protocols were used (Sambrook and Russell, 2001).

3.2.2 Polymerase chain reaction (PCR)

For amplification of the selected DNA sequences, such as cDNAs or genomic DNA, a 25 µl or 50 µl PCR reaction in a 0.2 ml tube was carried out. Depending on the manufacturer and application, PCR buffer and polymerase were according to manufacturer's instructions. For the colony PCR, an *E. coli* colony was removed with a yellow pipette tip. Some of the colonies to be tested were resuspended in the PCR reaction. A polymerase chain reaction contains following components:

Volume	Component	Final concentration
X µl	PCR-Buffer	(10x)
2 µl	dNTP-Mix	(10 mM; 2,5 mM pro dNTP)
1 µl	Primer 1	(20 µM)
1 µl	Primer 2	(20 µM)
1 µl	Template DNA	(0.1-1 ng plasmid, 0.1-1 µg DNA) or colony)
0.25 µl	Polymerase	(5 U/µl)
x µl	ddH_2O	
y* µl	Final volume	

*The final volume depends on the further purpose of the PCR (screening, purification, etc.)

The DNA amplification program is as follows:

Step	Temperature	Time	Number of cycles
1	95 °C	5 min	1

2	95 °C	30 sec	
3	55 °C	30 sec*	35
4	72 °C	1 min**	
5	72 °C	2-5 min	1
6	4 °C	until gel loading	1

* The annealing temperature depends on the composition and amount of the primers used.

** The elongation time depends on the size of an amplified template and the polymerase used in the PCR. In PCR reactions, in which a high amplification accuracy was desired, such as in cloning, the peqGOLD Pwo DNA polymerase (PEQLAB, Erlangen, Germany) has been used. This has a proofreading activity, which significantly reduces errors during amplification. For the analysis of the reaction either a part or the entire sample was separated by gel electrophoresis. Purification of PCR products was carried out by "NucleoSpin Gel and PCR Clean-up" kit (Macherey Nagel, Düren, Germany) according to manufacturer's instructions.

3.2.3 Yeast Colony PCR

For amplification of plasmid DNA from *S. cerevisiae*, a yeast colony PCR was performed. At first, the following substances were combined:

Volume	Component	Final concentration
2.5 µl	PCR-Buffer	(10x)
2.5 µl	dNTP-Mix	(10 mM; 2.5 mM pro dNTP)
0.5 µl	Primer 1	(20 µM)
0.5 µl	Primer 2	(20 µM)
0.25 µl	Polymerase	(5 U/µl)
18.5 µl	ddH_2O	
25 µl	Final volume	

For each individual reaction, a very small amount of yeast colony to be tested was added. The DNA amplification program is as follows:

Step	Temperature	Time	Number of cycles
1	95 °C	5 min	1
2	95 °C	30 sec	
3	55 °C	30 sec*	35
4	72 °C	1 min**	
5	72 °C	5 min	1
6	4 °C	until gel loading	1

* The annealing temperature depends on the composition and amount of the primers used.

** The elongation time depends on the size of an amplified template and by the polymerase used in the PCR.

5 µl of the PCR product was analyzed on an agarose gel.

3.2.4 RNA isolation from *A. thaliana*

For the isolation of *Arabidopsis* RNA, the "NucleoSpin RNA Plant" kit (MachereyNagel, Düren, Germany) was used according to manufacturer's specifications. The quantity and quality of RNA was checked by photometric measurement and gel electrophoresis.

3.2.5 Reverse Transcription

After isolation of RNA the reverse transcription of RNA to cDNA was carried out. This was done with the "RevertAid H Minus First beach cDNA Synthesis" kit (Thermo Fisher Scientific, Rockford, IL, USA), wherein the provided oligo (dT) primer was used. The resulting first strand cDNA was either directly used in a PCR or stored until use at -80 ° C.

3.2.6 Annealing of oligonucleotides

To anneal single-stranded, complementary oligonucleotides, the molecules were mixed in a ratio of 1:1. They both were each present in a concentration of 100 µM. In the PCR machine, the samples were first heated for 1 min at 95 °C and were then cooled down by one degree per min to room temperature.

3.2.7 Agarose gel electrophoresis

The separation of the DNA fragments based on their size was carried out by gel electrophoresis. An agarose gel was prepared in 1xTAE to a concentration between 0.7 - 2 % (w/v), dissolved in the microwave and stained with 1/10,000 (v/v) ethidium bromide solution (10 mg/ml). In the horizontal electrophoresis chamber, gels are run at a voltage of 6 - 8 V/cm. In addition, a DNA standard ladder (1 kb DNA Ladder GeneRuler or MassRuler Low Range DNA Ladder, Thermo Fisher Scientific, Rockford, IL, USA) has been applied in order to determine the size of the fragments. Using the gel documentation system, the agarose gel was photographed under UV light.

3.2.8 Purification of DNA fragments

The "NucleoSpin Gel and PCR Cleanup" kit (Macherey Nagel, Düren, Germany) was used for the purification of DNA fragments from agarose gels or PCR reactions according to the manufacturer.

3.2.9 Photometric determination of the concentration of DNA

In order to determine the concentration of DNA, a NanoPhotometer (Implen, Munich, Germany) was used. The ratio of OD_{260}/OD_{280} can identify protein impurities. The value of a clean DNA should be greater than 1.8. The ratio of OD_{260}/OD_{230} shows contaminants such as phenol and guanidine-HCl, and it should be greater than 2.0.

3.2.10 Preparation and transformation of electrocompetent cells

For preparation of electrocompetent cells, 5 ml dYT medium was inoculated with a single colony of *E. coli* XL1 Blue MRF' and incubated overnight on a shaker at 37 °C. On the next day the whole culture was transferred into 500 ml dYT and incubated at 37 °C with shaking until an OD_{600} of 0.5 to 0.8. The culture was then incubated on ice for 30 minutes and centrifuged at 400 *g* at 4 °C for 30 minutes. The supernatant was decanted and the pellet was resuspended in 500 ml ice-cold 10 % glycerol. This step is repeated with 250 ml and 10 ml of 10% glycerol. The pellet is finally resuspended in 1 ml of ice cold 10 % glycerol and 50 µl aliquots were frozen in liquid nitrogen. They were stored at -80 °C.

For transformation of DNA, 50 µl competent *E. coli* cells are thawed on ice. From a ligation mixture, 2 µl were added to the cells. This was followed by an incubation of 5 minutes on

ice. The entire batch was placed in a pre-chilled cuvette and the electroporation program was 2.5 kV, 200 ohms and 25 microfarads. Immediately after this step, 500 µl of SOC medium were added and the cells resuspended. The transformation mixture was incubated for 30 min with shaking at 37° C. Various volumes were plated onto agar plates with appropriate antibiotics and incubated overnight at 37° C.

3.2.11 Plasmid preparation from *E. coli*

For preparation of larger amounts of DNA, such as for use in the transformation of parsley protoplasts, "NucleoBond Xtra Midi EF" or "NucleoBond Xtra Maxi" kit (both from Macherey-Nagel, Düren, Germany) has been used. For isolation of DNA in smaller quantity and greater purity, for example for sequencing, the "EasyPrep Pro Plasmid Miniprep" kit (Biozym) was used.

Isolation of plasmid was also prepared by the following protocol, modified from (Li, J. F. et al., 2010). On the day before, desired colonies were precultured in 3-5 ml of LB medium with the appropriate antibiotic. This was incubated at 37 °C with shaking overnight. On the next day 1.5 ml of this culture was taken and centrifuged in an eppendorf tube (15,000 *g*, 1 min). After the supernatant was removed, the pellet was resuspended in 100 µl of solution A. This was followed by the addition of 100 µl of solution B and the tube was inverted 5 times. After 2 min, 100 µl solution C were added for neutralization and the tube was inverted gently for 5 times. The cell debris and genomic DNA were then centrifuged (5 min, 15,000 *g*). The supernatant was mixed with 500 µl solution D, then mixed with 20 µl silica suspension for the binding of DNA to the silica particles, mixed by vortexing, then the suspension was incubated 5 minutes at RT. The particles were collected by centrifugation (15,000 *g*, 10 s). The supernatant was discarded. By re-suspending of the pellet in 500 µl solution E, the silica particles were freed of protein residues. This washing step was performed twice. The wash solution was completely removed by pipetting off and the pellet was dried for 3 min at RT. The pellet was then resuspended in 40 µl ddH_2O and incubated for 2 min at 70 °C, whereby the plasmid DNA was released from the silica particles. After a final centrifugation step (15,000 *g*, 2 min) the plasmid-containing supernatant was isolated from the silica pellet and transferred into a new eppendorf tube. The concentration of the plasmid DNA isolated using this method was typically about 100-200 ng/µl.

3.2.12 Plasmid preparation from *S. cerevisiae*

In order to isolate a smaller amount of plasmid DNA from yeast, the alkaline lysis method was used. A yeast colony from the plate was put into 100 µl of cell suspension buffer (A) and incubated for 60 min at 37 °C. The vessel was inverted every 10 min. After the addition of 200 µl denaturing solution (B) the sulution was mixed by inverting repeatedly and incubated to lyse the cells for 5 min on ice. Then 150 µl renaturation solution (C) was added, carefully mixed and incubated for 5 min on ice. Centrifuge at 13,000 *g* for 5 min to separate the genomic DNA from the plasmid-containing supernatant from the cell debris. Subsequently, DNA was precipitated by adding a two-fold volume of 100 % ethanol and incubated at least for 15 min at -20 °C. After centrifugation at 13,000 *g* for 15 min, the resulting pellet was washed twice with 200 µl of 70 % ethanol. Centrifuge at 13,000 *g* for 5 min. After the ethanol was removed by pipetting and the pellet was allowed to air dry, DNA was dissolved in 15 µl LTE. Concentration of the isolated plasmid DNA was very low and was directly used to amplify by PCR. If a higher concentration is desired, a re-transformation of the plasmid into E. *coli* should be done.

3.2.13 Sequencing of DNA

In order to verify the accuracy of cloning or to analyze the amplified cDNA clones in Y1H screen, sequencing was performed by GATC Biotech (Konstanz, Germany). The data were analyzed with CLC Main Workbench software (CLC bio, Aarhus, Denmark). DNA and protein sequences obtained through www.arabidopsis.org or www.ncbi.nlm.nih.gov were used for comparison.

3.2.14 Storage of clones for long term use

For long term use, stocks of bacterial clones were created by mixing 700 µl of an *E. coli* culture (in LB with appropriate antibiotics) and 300 µl of glycerol, then freezing and storing at -80 °C.

For permanent storage of yeast clones, a fresh colony of the appropriate strain from a plate was isolated into 500 µl YPAD + 25 % glycerol (v/v), resuspended, frozen and stored at -80 °C.

3.2.15 Cloning of recombinant constructs containing synthetic promoters

For cloning of synthetic promoter constructs prior to transformation into parsley protoplasts, vector pBT10GUS-d35SLUC (Koschmann et al., 2012) was used. The desired single-stranded oligonucleotides were annealed and phosphorylated. The resulting SpeI/XbaI overhangs were ligated into the previously cut (SpeI/XbaI) and dephosphorylated pBT10GUS-d35SLUC vector. Before further recombinant work, the correct orientation and sequence of the inserted oligonucleotide was checked. Creating dimers and tetramers of oligonucleotide was done as described by (Koschmann et al., 2012).

The following primer pairs were used for screening of positive clones: the respective forward primer of each oligonucleotides and GUS75 reverse primer to check the orientation of the inserted monomers; M23 GUSLUC_r and M23 GUSLUC_f primers to test the presence of dimers and tetramers (Appendix, Table 12).

3.2.16 Cloning of recombinant constructs containing native promoters

To investigate *cis*-elements of the native *WRKY30* promoter in detail, different promoter fragments were cloned into the pBT10GUS-d35SLUC vector. For this, however, the minimal promoter had to be removed from the reporter plasmid. First, genomic DNA from *A. thaliana* was isolated for the amplification of the promoter fragments (NucleoSpin® Plant II; Macherey-Nagel, Düren, Germany), which acted as a template in the PCR. Depending on the size of the desired fragments, different forward primers (Appendix, Table 12) were used upstream of the translational starting site. Restriction sites were inserted into primers for cloning. The PCR was carried out by a proofreading polymerase. The vector was first digested with SpeI and then dephosphorylated. Since pBT10GUS-d35SLUC vector has two XhoI sites (positions 4706 and 4786), an incomplete digestion of the plasmid was necessary to remove the minimal promoter between position 4708 (SpeI) and position 4786 (XhoI). After gel electrophoretic separation, the 6,900 bp fragment (the remaining vector after digestion) was purified by phenol-chloroform and ligated with the PCR products. For ligation, the double amount of the digested vector had to be used, because the size difference between SpeI and SpeI/XhoI digested vector fragments is too low. The verification of positive clones was done by colony-PCR using the GUS75- reverse primer

and the respective forward primers. Desired clones were verified by sequencing for their accuracy and stocks were kept in LB-glycerol for permanent use.

The desired fragments of the native *WRKY30* promoter were amplified by the primers S1_SpeIF_230bp ; S2_SpeIF_217bp; S3_SpeIF_201bp; S4_SpeIF_155bp; S5_SpeIF_120bp and S_pro_XbaI-R (Appendix, Table 12) and cloned into pBT10GUS-d35SLUC_D that contains no TATA box and pre-digested by SpeI/XbaI.

3.2.17 Cloning of recombinant constructs for yeast one hybrid screenings

In order to examine sequences such as seq22 and 24 in the yeast one-hybrid system, they first had to be cloned into vector pHis2.1. These were amplified by proofreading polymerase from the recombinant pBT10GUS-d35SLUC with primers Bait_right GUSLUC / Bait_left GUSLUC (Appendix, Table 12) which incorporate an EcoRI and SacI site into the fragment. The fragment was cleaved and ligated into the appropriately cut vector pHis2.1. The verification of the construct was performed by sequencing.

3.2.18 Cloning of transcription factors in pCR2.1

Transcription factors selected in the yeast one hybrid screen were cloned by RT-PCR. After PCR, the cDNA of a transcription factor should be first inserted into a PCR cloning vector before transferring to the expression vector. First, RNA was isolated from *Arabidopsis* Col-0 plants and used in an RT-PCR to synthesize cDNA. Specifically designed primers, AtWRKY26-F and -R, AtWRKY40-F and -R, AtWRKY41-F and -R (Appendix, Table 12) for transcription factors with certain restriction sites were used to amplify cDNA by proofreading polymerase. The "TA Cloning" kit (Life Technologies, Darmstadt, Germany) was used for cloning the PCR product into the pCR2.1 vector. The positive clone was verified by sequencing.

3.2.19 Creation of T-DNA constructs for overexpression of transcription factors

To create a vector overexpressing the transcription factors selected by yeast one hybrid screenings in the plant system, the T-DNA vector pORE-O2-d35S-pA was used (Machens et al., 2014).

WRKY26-pORE-O2d35S-pA and WRKY40-pORE-O2d35S-pA.

WRKY26 or WRKY40 were excised from the corresponding recombinant pCR2.1 with BamHI/KpnI and ligated into the BamHI/KpnI digested pORE- O2d35S-pA vector.

WRKY41-pORE-O2d35S-pA

WRKY41 was excised from the corresponding recombinant pCR2.1 with SacI/KpnI and ligated into the SacI/KpnI - opened pORE-O2d35S-pA vector.

WRKY70-pORE-O2d35S-pA is described by Machens *et al*. (2014).

3.2.20 Transformation of *S. cerevisiae*

Transformation into yeast cells was modified from (Gietz and Schiestl, 2007). The Y1H-Gold strain (Clontech) was streaked out on a plate with a suitable medium (such as YPDA, SD dropout medium) and incubated for 3-5 days at 30 °C. Thereafter, a large single colony from the plate was transferred into a liquid medium and shaken overnight at 30 °C (200 rpm). Cell density was determined with a Thoma chamber. $2{,}5x10^8$ cells were added to 50 ml preheated 2xYPDA medium. The yeast cells were incubated with shaking (200 rpm) for about 4-5 hours up to a cell density of $2x10^7$ per ml. The cells were pelleted in a centrifuge for 5 min at 3,000 *g* and then resuspended in 25 ml of sterile ddH_2O for washing. The pellet was washed a second time, centrifuged, suspended in 1 ml of ddH_2O and transferred to a 1.5 ml Eppendorf tube. Centrifuge (30 sec, 13,000 *g*), and remove the supernatant and resuspend the pellet in 1 ml of sterile ddH_2O. 100 µl of this suspension was added to a 1.5 mL Eppendorf tube. These cells were pelleted (30 sec, 13,000 *g*) and the supernatant was completely removed with a pipette, then the following components were added on to the pellet as follows:

Volume:	Solution:
240 µl	50 % (w/v) PEG 3350
36 µl	1 M lithium acetate, pH 7.0
50 µl	SS carrier DNA (2 mg /ml)
34 µl	Plasmid DNA (0.1-1 µg) plus H_2O
360 µl	Final volume

Vortex thoroughly for 30 sec. This was followed by an incubation at 42 °C in a water bath for 40 min. The yeast cells were spun down at 13,000 *g* and resuspended in 1 ml of sterile

ddH_2O. The cells were plated on an appropriate selection medium (20-200 µl) or, if an increased yield was desired (such as in a Y1H screen), the cells were incubated at 30 °C in 1 ml 2xYPDA under shaking for 90 min prior to use.

3.2.21 Analysis of the bait strain

In order to analyze the background activity of the created strain, two clones of this strain in the appropriate selection medium that also lacks histidine were tested. In addition, the growth was tested with various concentrations of 3-AT (3-amino-1,2,4-triazole; 50 mM, 100 mM). The colony of the yeast cells was resuspended in 1 ml of sterile ddH_2O to create dilutions of 10^{-2}, 10^{-3}, 10^{-4}. 10 µl of these dilutions were dropped on the selection plates (Figure 2).

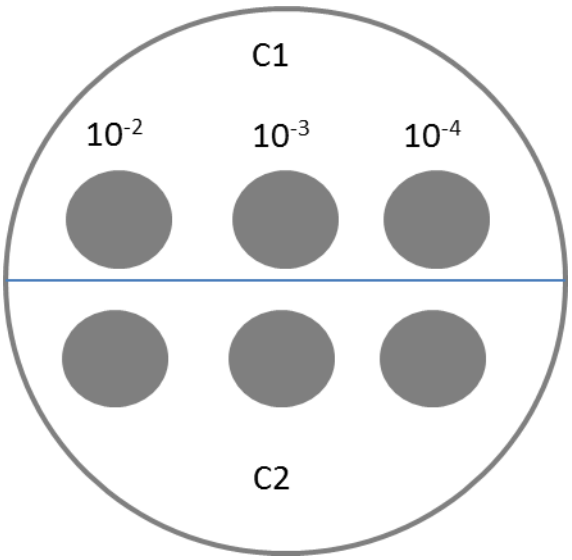


Figure 2. Drip scheme of 3-AT analysis of the bait strain

C1 = clone 1; C2 = clone 2; 10^{-2}, 10^{-3}, 10^{-4} dilutions of each clone.

After the plates were air-dried, they were incubated for 3 days at 30 °C. Based on the growth of the cells on the respective 3-AT-concentration, the concentration for the inhibition of the background was determined. This could be used in screening.

3.2.22 Yeast one-hybrid screening

To determine the transformation efficiency after transforming the library of *A. thaliana* transcription factors (Mitsuda et al., 2010) into the bait strains, 10 µl and 20 µl of cells were plated on SD -Trp/Leu selection medium. Thus, these plates only selected for the presence of two plasmids (Prey plasmid = Leu; Bait plasmid = Trp) but not for the

interaction between transcription factors and the sequence of the bait strain. The remaining cells were plated on SD -Trp/Leu/His + 3-AT selection plates. For this purpose, 500 µl of the suspension was plated on a large plate (150 mm diameter). These plates are selective for those that express the reporter gene.

The incubation of the plates at 30 °C was followed for 3-5 days. Cell growth was monitored every day and fast growing colonies marked. These were preferably used for further analysis. After a successful incubation, the labeled clones were inoculated onto fresh selection medium. These plates were again incubated at 30 °C for 2 days and then colonies were transferred to a new selection plate. This replica plating procedure was performed twice because it serves as a better selection of positive clones. After successful selection, the positive clones were checked by PCR using pDEST primers, and then analyzed by sequencing.

3.2.23 Analysing selected cDNA colones from the yest one-hybrid screening

Positive clones were investigated in more detail. Primers GAL4AD and GAL4AD-RV were used to amplify the cDNA of prey plasmids (Appendix, Table 12). The PCR products were separated by gel electrophoresis, which is already an initial indication of the specificity of the screens by comparing the cDNA lengths. Are many of the amplified products the same length, it is probably the same cDNA, wherein a heterogeneous picture represents often different cDNAs. After the analysis of the gel, the amplified products, which were to be sequenced were selected. For this, the PCR fragments were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) and then sent for sequencing. For sequencing primer Prey_attB_fwd was used (Appendix Table 12).

If a cDNA was selected several times, the interaction between transcription factor and Bait sequence was re-examined in the Bait strain through a retransformation of prey plasmid. For this purpose the plasmid DNA was isolated from the yeast clone and transformed in *E. coli* by electroporation. This was followed by plasmid isolation from *E. coli*. The prey plasmid was now transformed into the bait strain and growth was analysed on selective medium. As positive and negative controls for retransformation, p53His2 with pGADRec2-53 and pHis2.1 with pGADRec2-53 in Y1H-Gold were used, respectively (Clontech).

If growth over the 3-AT-limit of the bait strain is seen, it can be assumed that an interaction between the transcription factor of the prey plasmid and the sequence in the bait plasmid occurs. In this case, further investigations were carried out with these clones.

3.2.24 Parsley cell culture

The cultivation of parsley cell culture was carried out in 40 ml HA-medium in the growth chamber (CU32L) at 24 °C, 160 rpm and in the dark. After a every seven-day period, about 3 ml of the cell culture was transferred into a new 250 ml Erlenmeyer flask with 40 ml fresh HA-medium.

3.2.25 Transient reporter gene assays

3.2.25.1 Transient reporter gene assays in parsley protoplasts

Transformation of parsley protoplasts with recombinant pBT10GUS-d35SLUC harbouring tetramers of different *cis*-sequences and subsequent reporter gene assays were done as described earlier (Koschmann et al., 2012). Also, co-transformation experiments in parsley protoplasts with recombinant pORE-O2-d35S-pA expressing WRKY40, 41 and 70 as effector and recombinant pBT10GUS-d35SLUC as a reporter were done as described (Machens et al., 2014).

Parsley (*Petroselinum crispum*) callus culture Pc 5/3 was cultivated at 24 °C by shaking at 160 rpm in the dark. Subcultures were obtained by transferring 3 to 3.5 g of cells from a 7-day-old culture with a metal sieve into 40 ml of fresh HA medium. To isolate parsley protoplasts, a 5-day-old dark-grown parsley cell suspension culture was used. The culture was spun down at 300 g for 5 min at room temperature. The cell pellet was resuspended in 30 ml of enzyme solution and filled up to 90 ml with 0.24 M $CaCl_2$. Cells were shaken for 20 h at 20 rpm and 23 °C and subsequently for 20 min at 40-45 rpm at the same temperature in the dark. The suspension was divided into two 50 ml tubes and spun down for 2 min at 300 *g*. Cell pellets were washed with 20 ml of 0.24 M $CaCl_2$ (2 min, 300 *g*) each, resuspended in 25 ml of P5 medium each, and combined. After centrifugation (5 min, 300 *g*), the intact protoplasts float on the surface of the medium. These protoplasts where removed and transfered to a fresh 50 ml tube and filled up with P5 medium. This flotation procedure was repeated three times. Obtained protoplasts were used for transformation.

For transformation, 10 µg of plasmid DNA was mixed with 200 µl of PEG solution in a 15 ml tube. A total of 200 µl of protoplasts was added and mixed slightly. After incubation for 20 min in the dark, 5 ml of 275 mM $Ca(NO_3)_2$ supplemented with 2 mM MES (pH 6.0) was added to stop the transformation. Protoplasts were pelleted by centrifugation at 150 *g* for 7 min, and the cell pellet was resuspended in 6 ml of P5. Half of the suspension was transferred to a new 15 ml tube. Pep25 was added to one of the tubes (end concentration, 300 ng/ml). Protoplasts were incubated in the dark at 24 °C for 24 h. After this cultivation time, protoplasts were harvested by the addition of 9 ml of 0.24 M $CaCl_2$ and centrifugation for 10 min at 1,400 *g*. Cell pellets were frozen in liquid nitrogen and stored at -80 °C until GUS/LUC analysis.

3.2.25.2 Transient reporter gene assays in Arabidopsis protoplasts

For transient reporter gene assays in *Arabidopsis*, protoplasts were freshly isolated from 4-5 week old *A. thaliana* Col-0 plants cultivated under long day conditions using the Tape-Arabidopsis-Sandwich protocol (Wu et al., 2009). The isolated protoplasts were transformed according to Yoo and colleagues (Yoo et al., 2007).

Usually, 200 µl protoplasts ($4x10^4$) were transformed with a total amount of 30 µg plasmid DNA in a ratio 3:1 (reporter: transformation control). After transformation, the protoplasts were carefully resuspended in 2 ml 1x Gamborg B5 medium (Duchefa, Haarlem, The Netherlands) supplemented with 79.27 g/l glucose-monohydrate, 30 g/l sucrose, 0.1 mg/l 2,4-D and 0.015 mg/l 6-BAP. The protoplast suspension was divided and transferred into two wells of a 6-well culture plate. AtPep1 or flg22 was added to one of the wells to a final concentration of 2 nM or 1 µM, respectively. After incubation for 4 h (dark, 24 °C), protoplasts were harvested by addition of 1 ml W5 and centrifugation at 200 *g* for 2 min. The pellets were frozen in liquid nitrogen and stored at -80 °C until GUS/LUS analysis.

Protoplast lysis and subsequent determination of GUS and LUC activity was done as described before (Machens et al., 2014). In each experiment gene expression was determined by dividing the LUC activity through the normalized GUS activity. Values displayed in the figures are mean values from at least two independent experiments with two technical replicates each. The exact number of independent experiments for each construct is given in the figure legends. The error bar represents standard deviations.

3.2.26 Protein determination according to Bradford

The protein concentrations were determined according to Bradford (Bradford, 1976). The samples were diluted 1:10 or 1:50 in ddH_2O (depending on the protein concentration) and 50 µl of each diluted sample was pipetted in a transparent 96-well microplate in triplicates. In addition, a blank sample (sample buffer in appropriate dilution) was also used as control. 200 µl Bradford solution was added to each well and the 96 well microtiter plate was incubated for 5 min in the dark at room temperature. The absorption was measured with a TriStar LB 941 plate reader at 590 nm. The correction of values by the blank value was made automatically. In order to determine the protein concentration, different BSA concentrations (10-100 µg/ml; Carl Roth, Karlsruhe, Germany) were prepared in the sample buffer and absorption values were determined. A regression equation was generated from each standard curve. For every new 5x RotiQuant Bradford reagent (Carl Roth, Karlsruhe, Germany) or for each halogen lamp newly installed in the plate reader, a new standard series had to be established.

3.2.27 Quantitative LUC/GUS assay

LUC assay

A total of 150 µl of LUC extraction buffer was added to the frozen samples. After shaking (mixer 5432; Eppendorf) for 20 min at 4 °C, extracts were centrifuged for 10 min at 4 °C and 25,000 *g*. The supernatant was kept on ice and was used for protein quantification and GUS/LUC assays. Total protein was determined according to (Bradford, 1976), and a defined amount of 4 mg of protein in 50 ml of LUC extraction buffer was used for LUC assays. LUC assays were prepared according to (de Wet et al., 1987). Diluted samples were put into the wells of white 96-well microtiter plates, and the plates were inserted into the TriStar LB 941 micro plate reader (Berthold Technologies). A total of 50 ml of luciferin and 175 µl of LUC reaction buffer were added by the TriStar LB 941, and the luminescence was measured for 15 sec.

GUS assay

For the GUS analysis (Jefferson et al., 1987), 25 µl of the diluted protein extract (2 mg) was transferred into a well of a black 96-well microtiterplate. A total of 225 µl of GUS reaction

buffer was added, and the plate was inserted into the TriStar LB 941 and incubated at 37 °C for 10 min prior to measurements at 37 °C. For continuous measurement of GUS activity, the samples in each well were then measured every 15 min for 1 sec over the next 3 h (excitation, 360 nm; emission, 460 nm). Afterwards, for each well, a linear regression over the time period with a linear increase of fluorescence was performed. Nonlinear parts were excluded from the regression. The slope of the regression line was then transformed into pmol 4-MU/min/mg protein. For this, a calibration of fluorescence units with defined amounts of 4-MU was performed in the TriStar LB 941. A linear increase of fluorescence units with 4-MU concentration has been observed up to at least 75 mM. This linear correlation was then used for the transformation mentioned above. For each synthetic promoter, at least three independent experiments with two transformations each were carried out. To obtain comparable results that are independent from transformation efficiencies, all GUS values were normalized with the help of their corresponding LUC values. Only LUC values obtained without elicitor were used for normalization, because the same transformation gives lower LUC values after elicitor treatment, although the transformation efficiency should be the same. For normalization of the GUS values, one LUC value (without Pep25 elicitor) was selected and all other LUC values without elicitor were divided by this selected LUC value. The obtained quotients were used to divide corresponding GUS values with and without elicitor. Standard deviation (SD) values were calculated from these normalized GUS values. GUS values and SD values from controls (TATA and 4D) were calculated from all performed experiments.

In order to check whether the normalized values of the GUS-treated and untreated samples are significantly different to each other, an unpaired t-Test was carried out. A significant difference is observed at a value of $p \leq 0.05$ (5%). The normalized GUS values, standard deviations and significances are listed in the Appendix.

3.2.28 Cultivation of *A. thaliana* in sterile or soil culture

First approximately 1,000 seeds (about 20 mg) were subjected to sterilization for the culture of sterile *Arabidopsis* plants. Seeds were inverted in 70 % ethanol for 2 min, then the ethanol was replaced with 1 ml of sterilizing solution, and inverted for additional 8 min. Subsequently, the seeds were washed four times with sterile ddH_2O. Finally, the seeds were added to 0,1% agarose and then transferred by pipetting the desired number

on a ½ MS medium plate. After the plates were dried slightly under the clean bench, they were closed by Leukopor (BSN medical, Hamburg, Germany). The stratification of the seeds was carried out for 48 h at 4 °C in the dark. In a growth chamber (CU32L and CU-36L, Percival Scientific, Perry, IA, USA), the seeds or plants were incubated at 24 °C and under long-day conditions (16 h light, 8 h dark) or short-day conditions (8 h light, 16 h darkness) as required.

The growing of *Arabidopsis* plants in soil culture was initially done in a mixture of seeds and potting soil (1:1 mixture, Compo Sana, Münster, Germany). Without sterilization, they were sown on heavily watered soil and incubated for stratification at 4 °C in the dark for 48 h. Long-day or short-day conditions in a climatic chamber were used for cultivation. Watering of the soil was carried out in regular intervals. Waterlogging was avoided.

The propagation of the plants was carried out under long-day conditions, resulting in a flower sprout. Using the Aracon system (BETATECH, Ghent, Belgium), the flowering shoots could be covered while during seedpod develpment. After the complete maturation of the pods, the plants were no longer watered until the pods were fully dried. When the dried seeds were harvested, they were transferred into a paper bag and rubbed between two hands. Thus, the seeds could come out of the pod and are subsequently separated by a wire mesh screen from other plant debris. The dry seeds were stored at room temperature until use.

3.2.29 Gelshift-Assay

The gelshift experiments were performed with slight modifications according to the manufacturer's protocol "LightShift Chemiluminescent EMSA Kit" (Thermo Fisher Scientific, IL, USA) for analysis of protein-DNA interactions *in vitro*. The biotin-labeled oligonucleotides were generated according to the ´´Biotin 3´ End DNA Labeling Kit`` (Number 89818, Thermo Fisher Scientific, IL, USA).

The 6% acrylamide gel was prepared according to the standard protocol and casted in the "Mini Protean Tetra Cell" (Bio Rad, Munich, Germany) associated glass plates for mini-gels (7.3 x 10.1 cm, 1 mm spacer):

5.55 ml dH_2O

1.25 ml Rotiphorese® Gel 40 (29: 1, Carl Roth)

0.75 ml 5x TBE

75 µl 10% (w/v) APS

4.5 µl TEMED

Prior to loading with sample, a pre-run of the gel in 0.5x TBE for 30-60 min at 100 V was done.

For the binding reaction, purified recombinant protein was used in 1x gelshift binding buffer. For a typical binding reaction in 20 µl volume, the following components were added together in the order given below and carefully mixed:

ddH_2O	… µl
10x gel shift binding buffer	2 µl
Poly (dI-dC) [1 µg /µl]*	1 µl
Unlabeled competitor DNA [2-10 µM]	1 µl (depending on the experiment)
Recombinant protein (in 1x binding buffer)	0.1-0.5 µg
Biotin-labeled DNA [0.01 µM]	2 µl
Total volume	20 µl

*from LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, IL, USA)

Before the addition of the biotin-labeled DNA, a 10 min pre-incubation with competitor DNA was carried out (23 °C). Then the labeled probe was added for 25 min (23 °C). Subsequently, 5 µl 5x EMSA loading buffer (Thermo Fisher Scientific, IL, USA) was added and the probe was loaded on the 6 % polyacrylamide gel and electrophoresed at 100 V. The subsequent blotting of the gel on biodyne B nylon membrane (Thermo Fisher Scientific, IL, USA) was carried out with the "Mini Trans-Blot Cell" (BioRad, München, Germany) according to the instructions in the kit. Then the fixing of the membrane was performed with the "Stratalinker UV cross linker" (Stratagene, La Jola, CA, USA) with the auto crosslink function. The subsequent detection of biotin-labeled DNA was also performed according to the manual of the kit. The exposure time for the X-ray film was typically 1-10 min before it was developed.

4. Results

4.1. The presence of two W-boxes and one WT-box in CRM1 and CRM2 is important for the response of a synthetic promoter in parsley protoplasts to MAMPs

In the upstream region of the *WRKY30* gene, there are two MAMP-responsive *cis*-regulatory modules (CRMs) that were recently discovered: CRM1 (seq. 24) and CRM2 (seq. 22) (Koschmann et al., 2012; Machens et al., 2014). As presented in Figures 4 and 5, each of these modules contains two W-boxes and one WT-box.

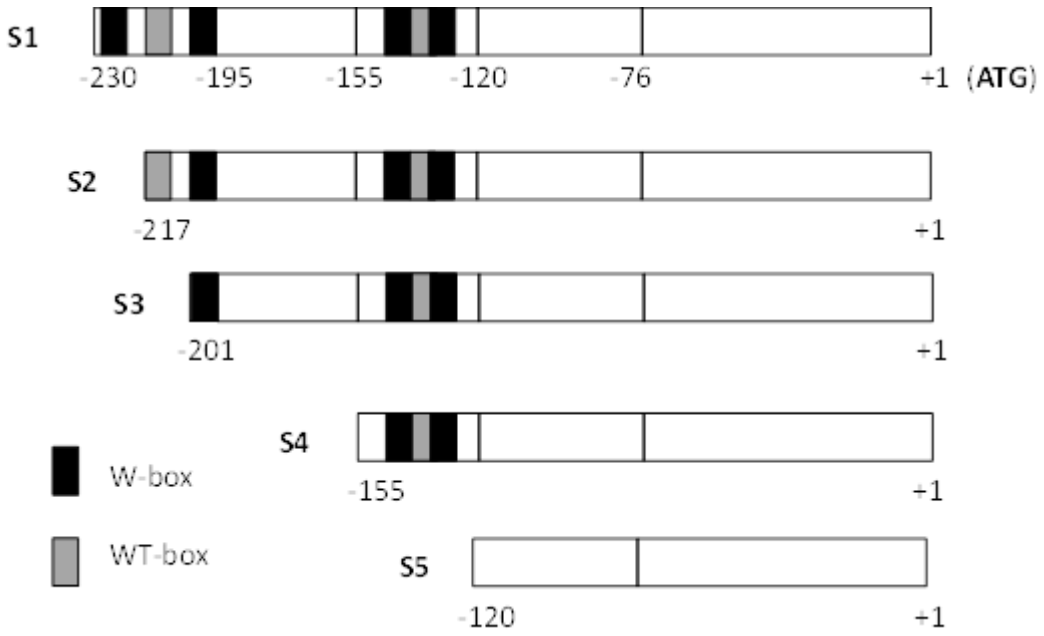


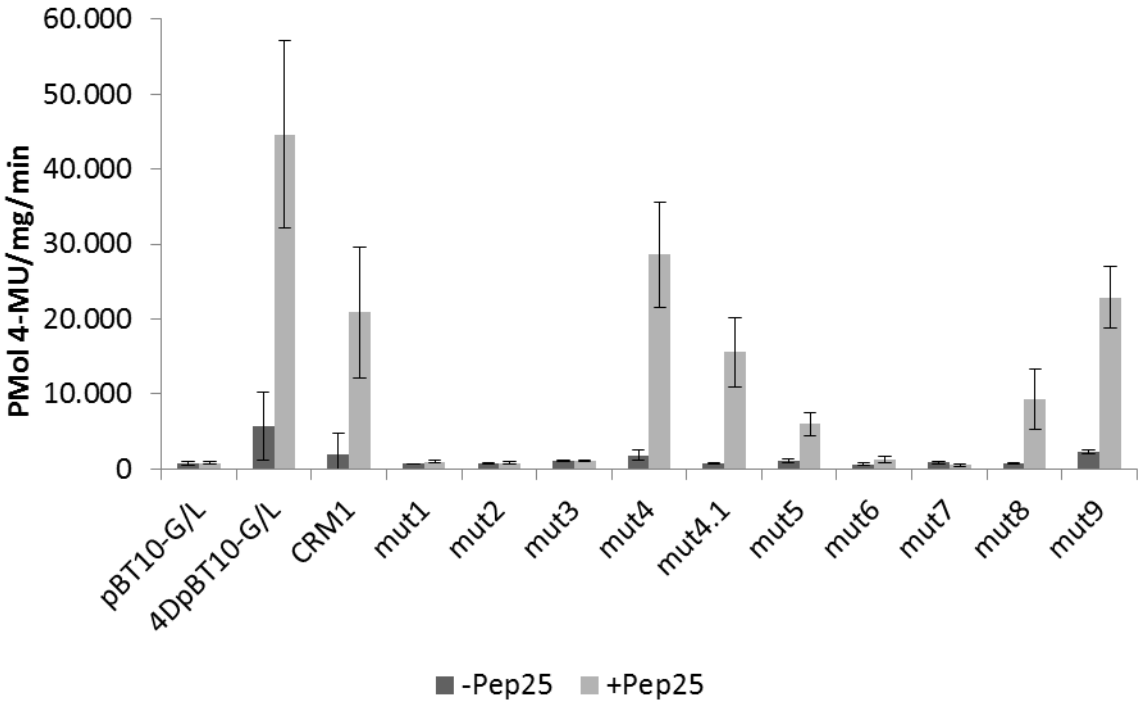
Figure 5. The WRKY30 promoter with the two *cis*-regulatory modules CRM1 and CRM2.

Schematic representation of 230 bp upstream of the translation start site of the *WRKY30* gene. The sequence and positions of the two CRMs are shown above the promoter. A solid line below the sequence indicates the W-boxes and a dashed line the WT-boxes in both CRMs. Constructs S1, S2, S3, S4, and S5 are 5' promoter deletions tested for MAMP responsivity in parsley protoplasts. W- and WT-boxes of the CRM1 and CRM2, endpoints of promoter deletions, and the transcription start site (-76) relative to the start codon (+1) are indicated.

In order to determine the role of these W- and WT-boxes in the response of parsley protoplasts to induction by microbial signals, we generated different mutations in CRM1 and CRM2 (Figures 6B and 7B) and tested the expression of pBT10GUS-d35SLUC habouring

tetramers of the mutated sequences upstream of the *uid*A (GUS) gene (Figure 6A and 7A). Pep25, an oligopeptide from the surface glycoprotein of *Phytophthora sojae* (Nürnberger et al., 1994; Rushton et al., 1996), was used as an inducer. The enhancerless *uidA* gene in pBT10GUS-d35SLUC was used as negative control (pBT10). pBT10GUS-d35SLUC containing four copies of the D-box (4D) from the parsley *PR2* promoter was used as a positive control (4DpBT10) (Koschmann et al., 2012). In Figure 6 and Figure 7, the activity of the GUS reporter gene for Pep25-treated or untreated cells transformed with the respective plasmid are presented after normalization with luciferase expression (LUC). High activity of the reporter gene in the positive control as well as the CRM1 after Pep-25 treatment shows the successfulness of our assay.

(A)



(B)

```
CRM1     TGGTCAGCATGTTGGACTTTCCAAATTCATTGACC
mut1     -------------AAGTCCCTT-------CCAGTT
mut2     -AACTG----------------------CCAGTT
mut3     -AACTG-------AAGTCCCTT-------------
mut4     ----------------------------CCAGTT
mut4.1   -----------------------------CAGT-
mut5     -------------AAGTCCCTT-------------
mut6     -AACTG-----------------------------
mut7     -AACTG-------AAGTCCCTT-------CCAGTT
mut8     ------ATGCACC----------------------
mut9     ---------------------GGGCCTG------
```

Figure 6. Mutation analysis of the CRM1-WRKY30 for MAMP-responsive gene expression in parsley protoplasts.

(A) Transient reporter gene assays after parsley protoplast transformation and treatment with and without Pep25. The plasmids used for transformation harbour the CRM1 and ten mutations of the CRM1 in four copies upstream of the *uidA* reporter gene in pBT10GUS-d35SLUC. The empty vector pBT10GUS-d35SLUC (pBT10) and pBT10GUS-d35SLUC with four copies of the D-box upstream of the *uidA* reporter gene (4DpBT10) serve as negative and positive controls, respectively. The number of experiments was 3 (pBT10, 4DpBT10, mut4, 5, 6, 7), 5 (CRM1), and 2 (mut1, 2, 3, 4.1, 8, 9). In each experiment GUS expression was measured twice. The mean values and standard deviations were determined from all measurements (see Table 4, Appendix). (B) The sequence of the CRM1 and that of ten mutations. Altered nucleotides in the sequences are shown, unaltered nucleotides are not shown (-). The two W-boxes and the WT-box are underlined.

Figure 6 shows that double mutations in the W-boxes and/or WT-box as well as a triple mutation (mut1, mut2, mut3, mut7) nearly diminish MAMP-responsive gene activity, indicating that both W-boxes and the WT-box in CRM1 are critical for the regulation of gene expression. The double mutations also show that a single W-box or WT-box is not sufficient for MAMP-responsive gene activity and suggests that at least two *cis*-sequences are required for MAMP-responsive gene activity. This was further analysed with single mutations in each of the W- and WT-boxes. Indeed, when single mutations in the second W-box (mut4.1) or the WT-box (mut5) were introduced, GUS-activity was also reduced but to a lesser extend compared to double mutations. It is still unclear why a similar mutation in the second W-box (mut4) shows no reduction of GUS-activity at all. However, notably, a

single mutation in the first W-box (mut6) almost leads to abolishment of GUS-expression. Comparing these single mutations, it was clear that the presence of the first W-box in CRM1 is the most important element for MAMP-responsiveness. It also indicates that the first W-box is not sufficient for the full upregulation of the reporter gene which needs all W- and WT- boxes. Moreover, a decrease of GUS activity in mut8 pointed out that the nucleotide sequence between the first W-box and the WT-box also contributes to the MAMP-response of CRM1. In contrast, amongst all tested mutated sequences, mutation in the region between the WT-box and the second W-box (mut9) shows comparable level of GUS to that of the unmutated CRM1. Thus, this nucleotide sequence shows no effects on the response of parsley protoplasts to Pep25.

Similarly, mutations in CRM2 were generated to test the importance of different elements in this module (Figure 7). A single mutation in the first W-box reduced the expression of GUS (mut1) but to a lesser extend compared to a mutation in the second W-box (mut2) or double mutations in both W-boxes (mut3). Therefore the presence of the second W-box in CRM2 may be more important than the first one. To check the role of the WT-box in CRM2, mut4 was generated and showed almost no MAMP-responsiveness. Because the WT-box and both W-boxes overlap (Figures 4 and 5), it is still unclear whether the WT-box itself or both of the adjacent W-boxes contributed to this reduction. Therefore a mutation only affecting the WT-box was generated. Mut5 also showed a strong decrease of the reporter gene activity, indicating the importance of the two nucleotides between the two W-boxes as well.

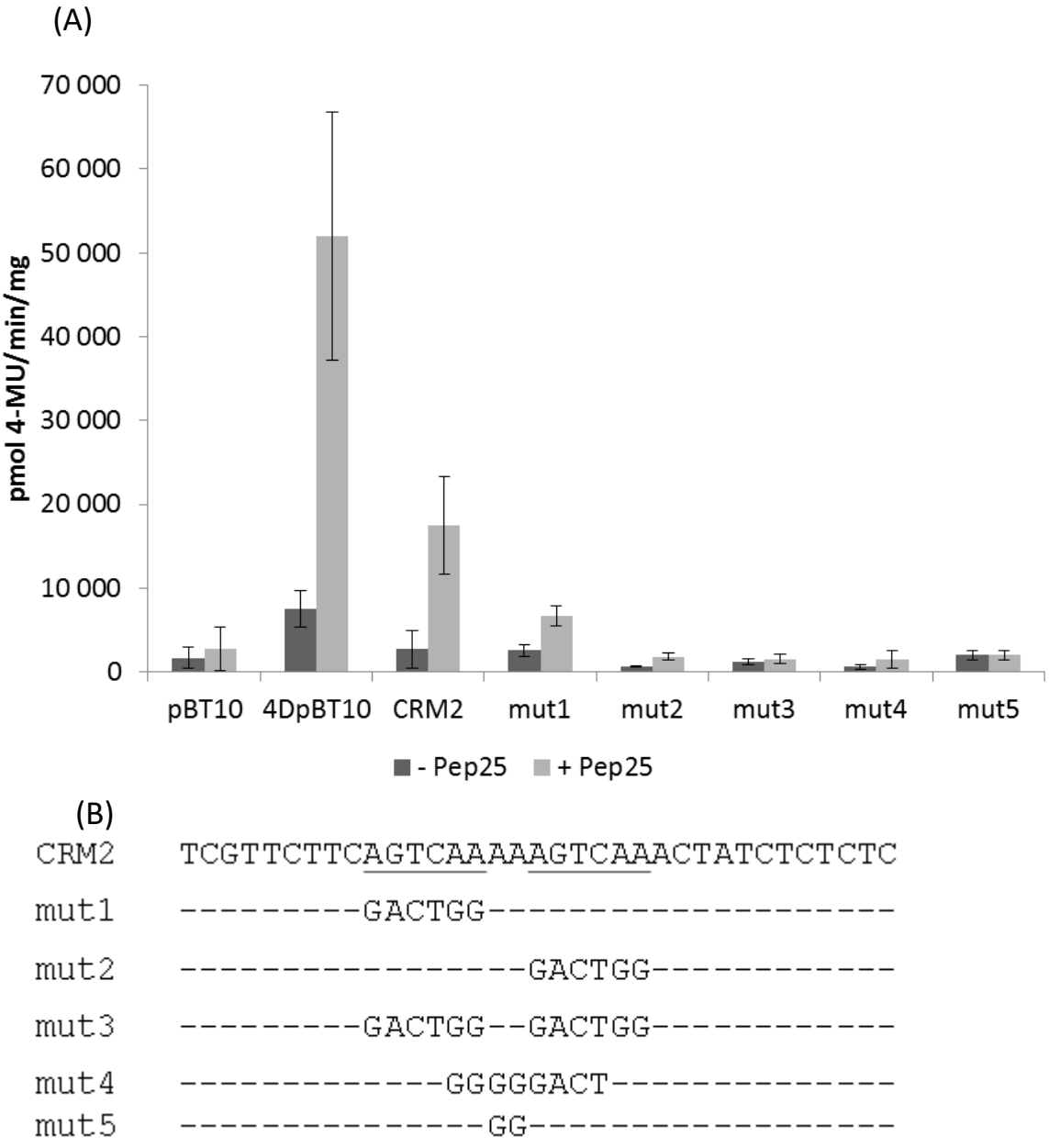


Figure 7. Mutation analysis of the CRM2-WRKY30 for MAMP-responsive gene expression in parsley protoplasts

(A) Transient reporter gene assays after parsley protoplast transformation and treatment with and without Pep25. The plasmids used for transformation harbour the CRM2 and five mutations of the CRM2 in four copies upstream of the *uidA* reporter gene in pBT10GUS-d35SLUC. Negative (pBT10) and positive (4DpBT10) controls are the same as in Figure 1. The number of experiments was 4 (pBT10, 4DpBT10 with pep25, CRM2) and 2 (4DpBT10 without pep25, mut1, 2, 3, 4, 5). In each experiment GUS expression was measured twice. The mean values and standard deviations were determined from all measurements (Table 5, Appendix). (B) The sequence of the CRM2 and that of five mutations. Altered nucleotides in the sequences are shown, unaltered nucleotides are not shown (-). The two W-boxes are underlined.

4.2. CRM1 is required for MAMP responsivity of the *WRKY30* promoter in parsley protoplasts

The functions of W-boxes and WT-boxes in either CRM1 or CRM2 were analysed using tetramers of the respective module. In the next step, we aimed to determine the function of these CRMs in context of the native promoter, i.e. only a monomer of the *WRKY30* promoter region was cloned into the reporter gene vector. We designed different constructs containing deletions of components in CRM1 and CRM2 (Figure 5). S1 is the wildtype promoter that contains a full set of W-boxes and WT-boxes from CRM1 and CRM2. The first W-box of CRM1 is absent in S2 and a further WT-box is absent in S3. Both CRM1 and the nucleotide region upstream CRM2 are deleted in S4. In S5, CRM1 and CRM2 (from nt. -230 to nt. -120 upstream of the first ATG) are missing. These promoter fragments were cloned upstream of the *uid*A gene in plasmid pBT10GUS-d35SLUC after removing the CaMV minimal promoter, transformed into parsley protoplasts, and reporter gene activity was measured after Pep25 treatment as previously reported (Koschmann et al., 2012).

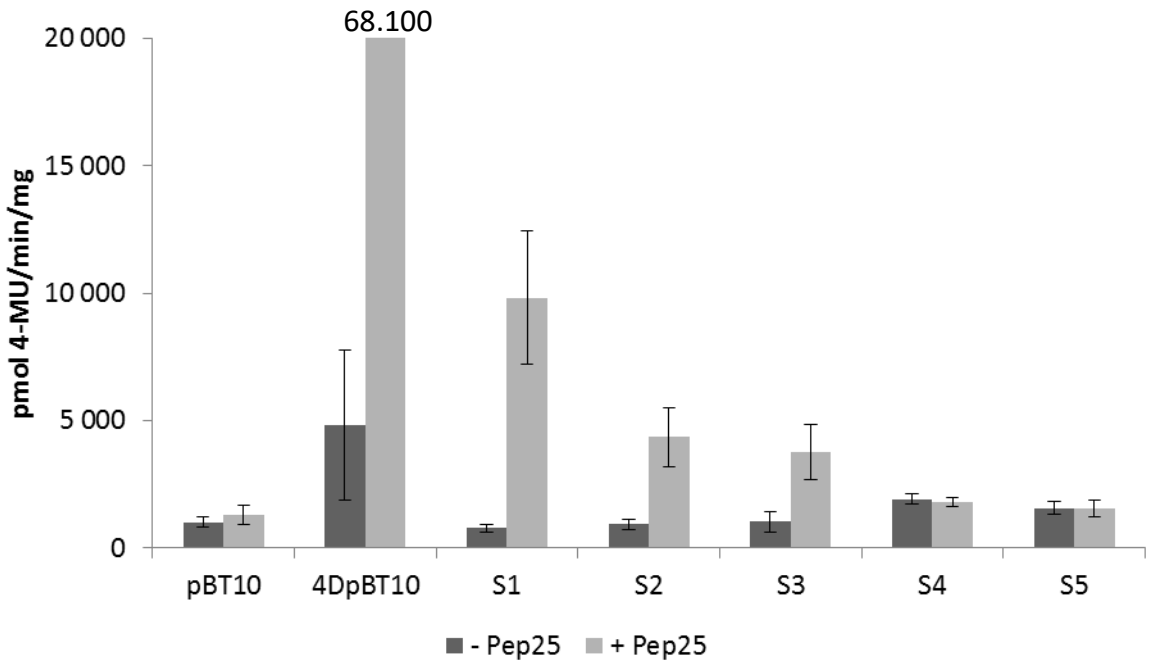


Figure 8. MAMP-responsive gene expression driven by WRKY30 promoter fragments requires CRM1.

Transient reporter gene assays after parsley protoplast transformation and treatment with and without Pep25. The plasmids used for transformation harbour five different promoter fragments (B) upstream of the *uidA* reporter gene in pBT10GUS-d35SLUC without the minimal promoter of the *uidA* gene. Negative (pBT10) and positive (4DpBT10) controls are the same as in Figure 1. The number of experiments was 3 (4DpBT10), 4 (pBT10, S1, 2, 3) and 2 (S4, 5). In each experiment GUS expression was measured twice. The mean values and standard deviations were determined from all measurements (Refer to Table 6, Appendix).

Figure 8 shows the successful assay in a context of the native promoter (S1). A sharp decrease of GUS activity was seen in S2, indicating the necessity of the first W-box of CRM1 for the response in parsley protoplasts to Pep25. A further deletion of the WT-box (S3) resulted in a similar level of GUS activity compared to S2. These data show that the first W-box of CRM1 is more important for MAMP responsivity than the WT-box. The deletion of the whole CRM1 cassette and upstream region of CRM2 led to a further decrease of Pep25 responsivity, meaning that CRM2 is not sufficient for the MAMP response (S4, compared to S2 and S3). Likewise, the deletion of both CRM1 and CRM2 (S5) led to a similar GUS activity comparable to S4 that only harbours CRM2. Only the native promoter (S1) (Figure 8) shows high GUS expression that was nearly 50% compared to that of the tetrameric CRM1 in the synthetic promoter (Figure 6) and 75% compared to the tetrameric CRM2 in the synthetic promoter (Figure 6). In summary, these results show that CRM1 is required for MAMP responsivity of the *WRKY30* promoter while CRM2 alone is not sufficient.

4.3. CRM1 but not CRM2 are flg22- and AtPep1-responsive in *A. thaliana* protoplasts

The functionality of the CRMs from the WRKY30 promoter in the parsley protoplasts was described above. In this part, we want to determine the roles of these CRMs in *A. thaliana* cells as its system of origin. Because transformation of pBT10GUS-d35SLUC containing CRM1 or CRM2 into *A. thaliana* protoplasts led to high background of the reporter gene, we could not use this vector. Thus, tetramers of either CRM1 or CRM2 were isolated from recombinant pBT10GUS-d35SLUC plasmid and cloned into pBT10LUC (Material and Method). A second plasmid expressing *uid*A (pRT103GUS) was co-transformed into *A. thaliana* protoplasts as a control for successful transformation. Thus, luciferase activity

was measured relative to GUS activity. The following data were provided by Mona Lehmeyer (personal communication).

In the context of *A. thaliana* protoplasts, two treatments were applied to test the MAMP responsivity of the promoter. Flg22 is a peptide (22 amino acids) isolated from the conserved N-terminus of flagellin which can induce a defense reaction in plants (Felix et al., 1999; Gómez-Gómez and Boller, 2000). AtPep1 contains 23 amino acids of the long C-terminus of *Arabidopsis* PROPEP1 which can induce early immunity of plants (Huffaker et al., 2006).

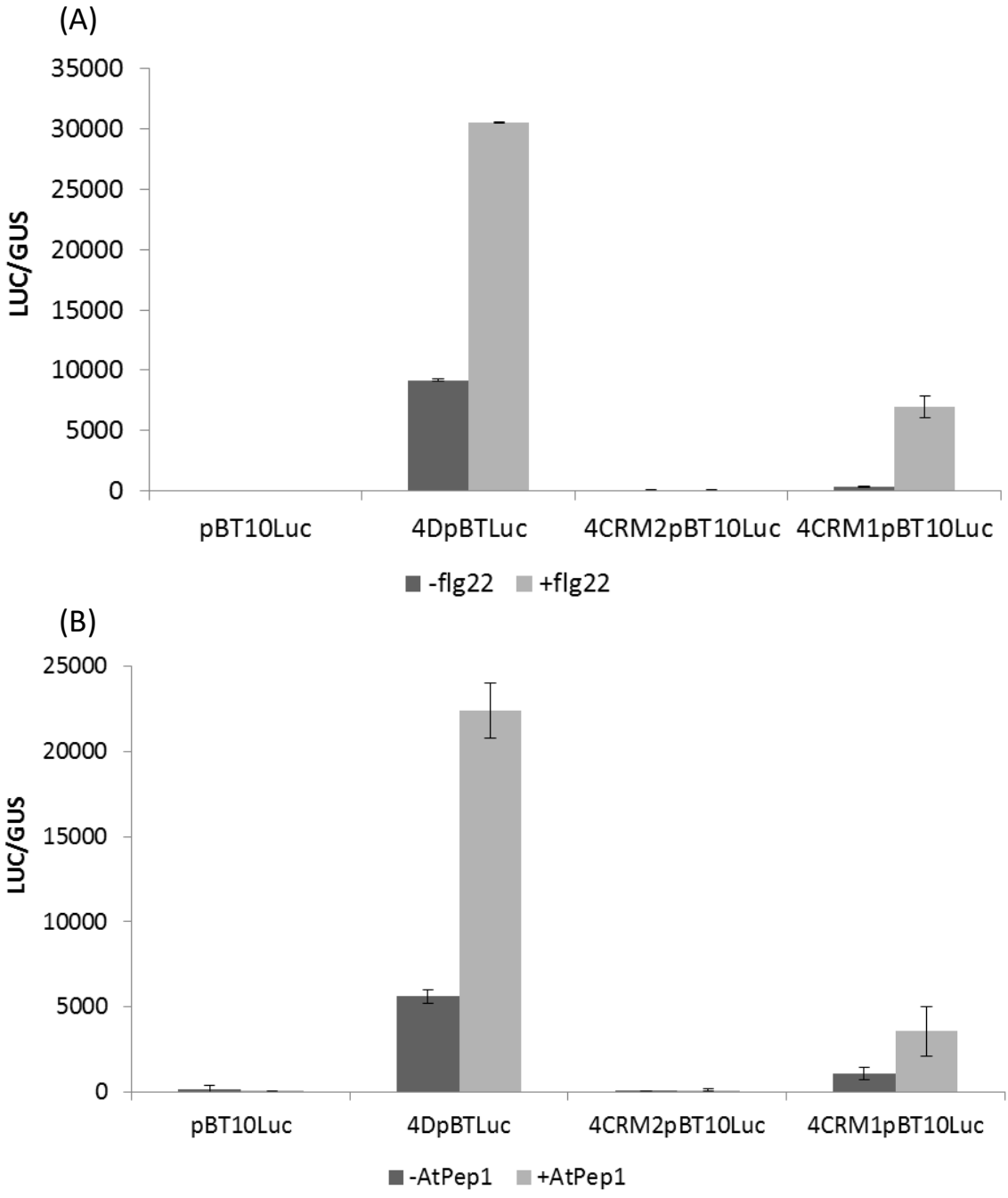


Figure 9. CRM1 but not CRM2 confers flg22 and AtPep1 responsive reporter gene activity *A. thaliana* protoplasts.

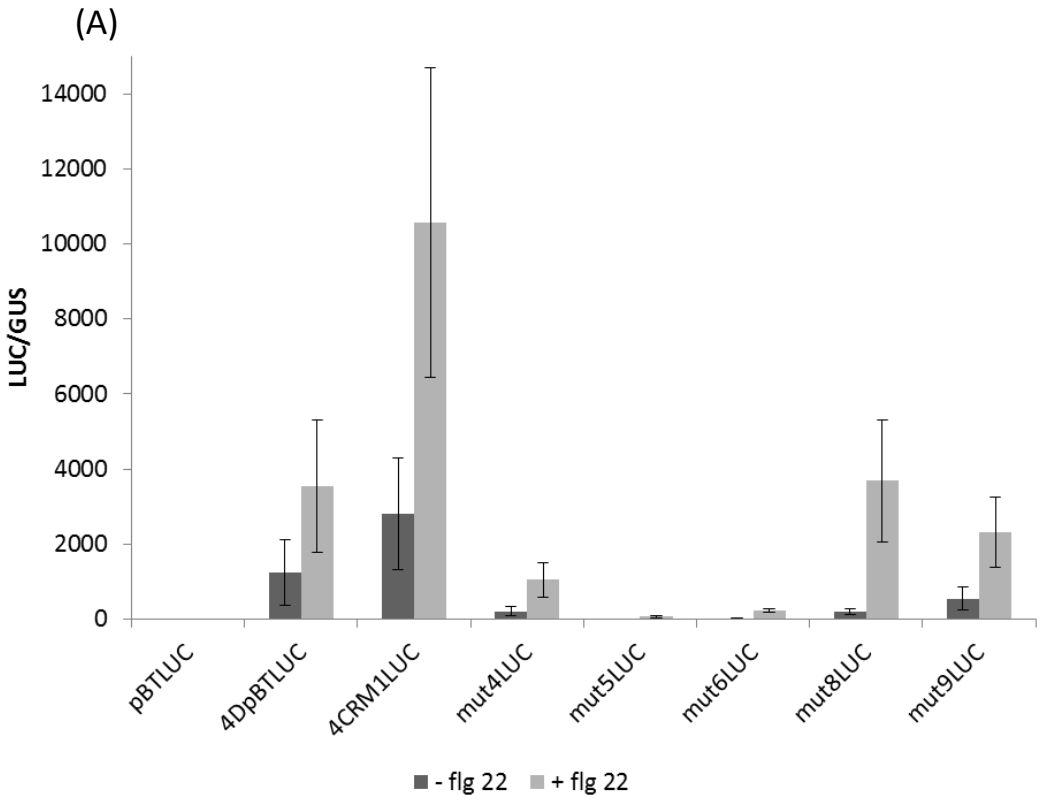
Transient reporter gene assays after *A. thaliana* protoplast transformation and treatment with and without flg22 (A) and AtPep1 (B). The plasmids used for transformation harbour four copies of the CRM1 and CRM2 upstream of the luciferase (LUC) reporter gene in pBT10LUC. The empty vector pBT10LUC and pBT10LUC with four copies of the D-box upstream of the LUC reporter gene (4DpBT10LUC) serve as negative and positive controls, respectively. The number of experiments was 2 for each plasmid and condition (+/-). In each experiment LUC expression was measured twice (Lehmeyer, personal communication). LUC expression was normalized by dividing through GUS expression from a cotransformed plasmid (pRT103GUS) constitutively expressing the GUS gene. The mean values and standard deviations were determined from all measurements.

The high gene expression of the positive control (4DpBTLUC) (Figure 9) shows the successful assay and allows the analysis of the synthetic constructs. After flg22 treatments, no reporter gene activity was seen when 4CRM2pBT10LUC was transformed (Figure 9A). In contrast, there was a clear response of 4CRM1 after flg22 treatment in *A. thaliana* protoplasts. Similarly, only 4CRM1 responded to AtPep1 while 4CRM2 was not responsive (Figure 9B). This result indicates that only CRM1 is MAMP-responsive in *A. thaliana* protoplasts.

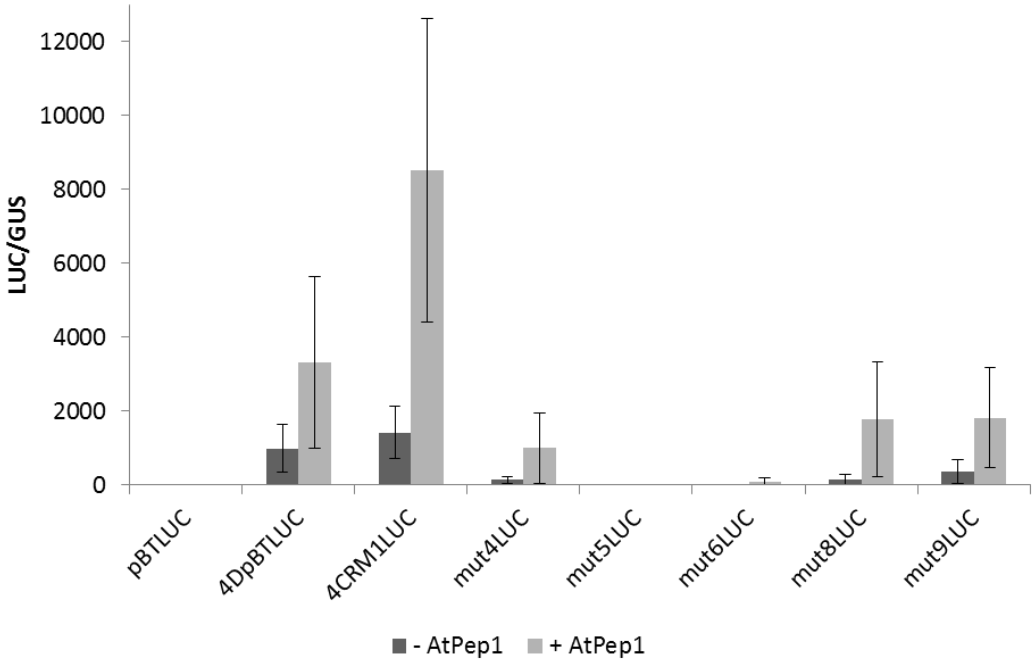
4.4. The WT-box and the two W-boxes are important for flg22 and AtPep1 responsivity of the CRM1

CRM1 was shown to be important for flg22 and AtPep1 responsivity in *A. thaliana* protoplasts. Therefore, we wanted to analyze the function of this module in *A. thaliana* in more detail. Single mutations in CRM1 (Figure 6) were re-cloned into pBT10LUC, designated as mut4LUC, mut5LUC, mut6LUC, mut8LUC and mut9LUC, then transformed into *A. thaliana* protoplasts to test the role of specific regions in the CRM1. As shown in Figure 10A and 10B, after flg22 and AtPep1 treatment, mutation in the second W-box of CRM1 (mut4LUC) led to a strong reduction of reporter gene activity compared to the unmutated control (4CRM1LUC). However, this reduction was still smaller than the reduction observed in mut5LUC and mut6LUC where the LUC/GUS activity was almost abolished. These results show that the first W-box of CRM1 is more important than the second W-box in *A. thaliana.* That is in line with the parsley protoplast data. However, in contrast to the parsley protoplast results that show less importance of the WT-box (Figure

6), our data in *A. thaliana* emphasized the equal participation of both WT-box and the first W-box (mut5LUC and mut6LUC, Figure 10 A and B). Moreover, our data also show the contribution of nucleotides between W-boxes and WT-box (mut8LUC and mut9LUC, Figure 10 A and B) in *A. thaliana* protoplasts. In contrast, mut9 had no effects in parsley protoplast (Figure 6). In summary, these results indicate the requirement of all WT- and W-boxes as well as the adjacent nucleotides in the CRM1 for the responsivity to flg22- and AtPep1.



(B)



(C)

```
CRM1    TGGTCAGCATGTTGGACTTTCCAAATTCATTGACC
mut4    ------------------------------CCAGTT
mut5    ------------AAGTCCCTT------------
mut6    -AACTG-------------------------------
mut7    -AACTG-------GGACTTTCC-------CCAGTT
mut8    ------ATGCACC----------------------
mut9    ----------------------GGGCCTG------
```

Figure 10. The W- and WT-boxes of CRM1 are required for flg22 and AtPep1 responsive gene expression.

Transient reporter gene assays after *Arabidopsis* protoplast co-transformation of two plasmids. One plasmid harbours the CRM1 and six mutations upstream of the LUC reporter gene in pBT10LUC and a second plasmid constitutively expressing the *uidA* reporter gene in pRT103-GUS as a transformation control. After transformation samples were treated with and without flg22 and AtPep1. Negative (pBT10LUC) and positive (4DpBT10LUC) controls are the same as in figure 5. (A) Normalized LUC values obtained after transformation and treatment with flg22 from four independent experiments. LUC expression was normalized by dividing through GUS expression from a co-transformed plasmid constitutively expressing the *GUS* gene (pRT103GUS). The mean values and standard deviations were determined from all experiments (Refer to Table 7, Appendix). (B) Normalized LUC values obtained after transformation and treatment with AtPep1 from three independent experiments. The mean values and standard deviations were determined from all experiments (refer to Table 8, Appendix). (C) The sequence of the CRM1 and that of six

mutations. Altered nucleotides in the sequences are shown, unaltered nucleotides are not shown (-). The two W-boxes and the WT-box are underlined.

4.5. Identification of TFs interacting with CRM1 and CRM2

In order to identify which TFs bind to CRM1 and CRM2 of the *WRKY30* promoter, a TF-only prey library with 1,500 different *A. thaliana* TFs (Mitsuda et al., 2010) was used for yeast one-hybrid screenings with both CRMs. The results are shown in Table 2. Both, CRM1 and CRM2, interact with WRKY factors. We observed that the interaction of WRKYs with CRM1 was more specific than with CRM2 because CRM1 only selected 7 different WRKYs and only two of them were selected once, whereas CRM2 selected 14 different WRKYs and ten of them were selected once. WRKY40 and WRKY53 showed the most frequent interaction with CRM1 (16 and 15 times, respectively). Although WRKY41 and WRKY70 were selected less frequently (3 and 6 times), they are more specific for CMR1 because they were not selected with CRM2.

Table 2. Number of WRKY factor expressing clones selected in a yeast one-hybrid screen with CRM1 and CRM2

Transcription factors	CRM1	CRM2
WRKY40	16	1
WRKY53	15	7
WRKY70	6	0
WRKY41	3	0
WRKY26	2	1
WRKY11	1	5
WRKY57	1	1
WRKY28	0	3
WRKY18	0	4
WRKY15	0	1
WRKY10	0	1
WRKY29	0	3
WRKY75	0	1

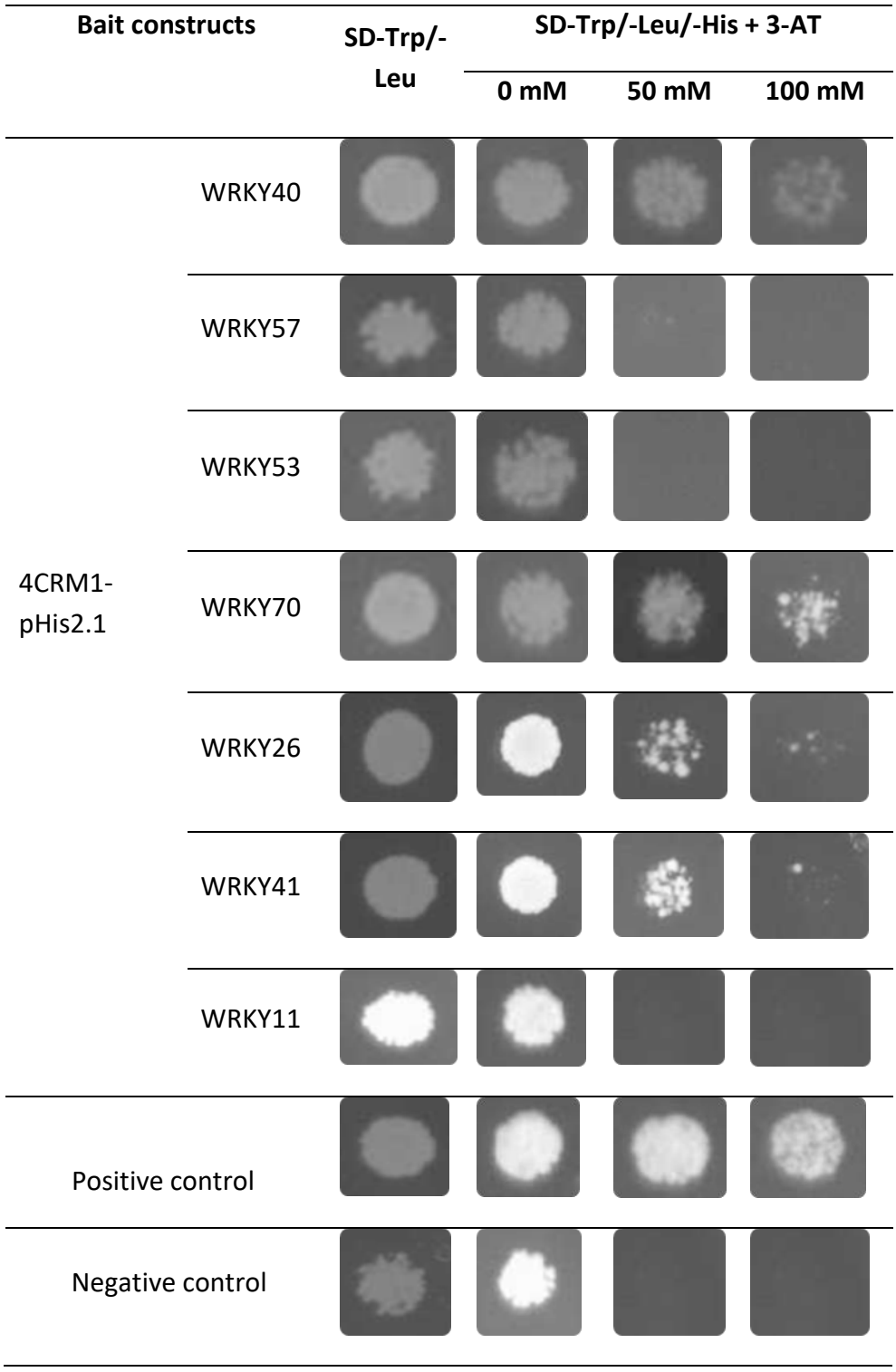
WRKY48	0	1
WRKY57	0	1
WRKY4	0	1
WRKY63	0	1

All selected WRKY clones of CRM1 were retransformed into a yeast strain containing the CRM1 bait construct to confirm the specificity of the interaction in the above screening. The assay was not done for the CRM2 bait construct because the above experiment already showed the stronger importance of CRM1 in the parsley (Figure 8) and *A. thaliana* system (Figure 9). Figure 11A shows that WRKY11, 53 and 57 are not selected on the SD-Trp/-Leu/-His medium containing 50 mM or 100 mM 3-AT. In contrast, WRKY26, 40, 41 and 70 allowed the growth of the yeast strain containing tetramers of CRM1 under 50 mM and 100 mM 3AT conditions; hence the following experiments focused on these 4 factors.

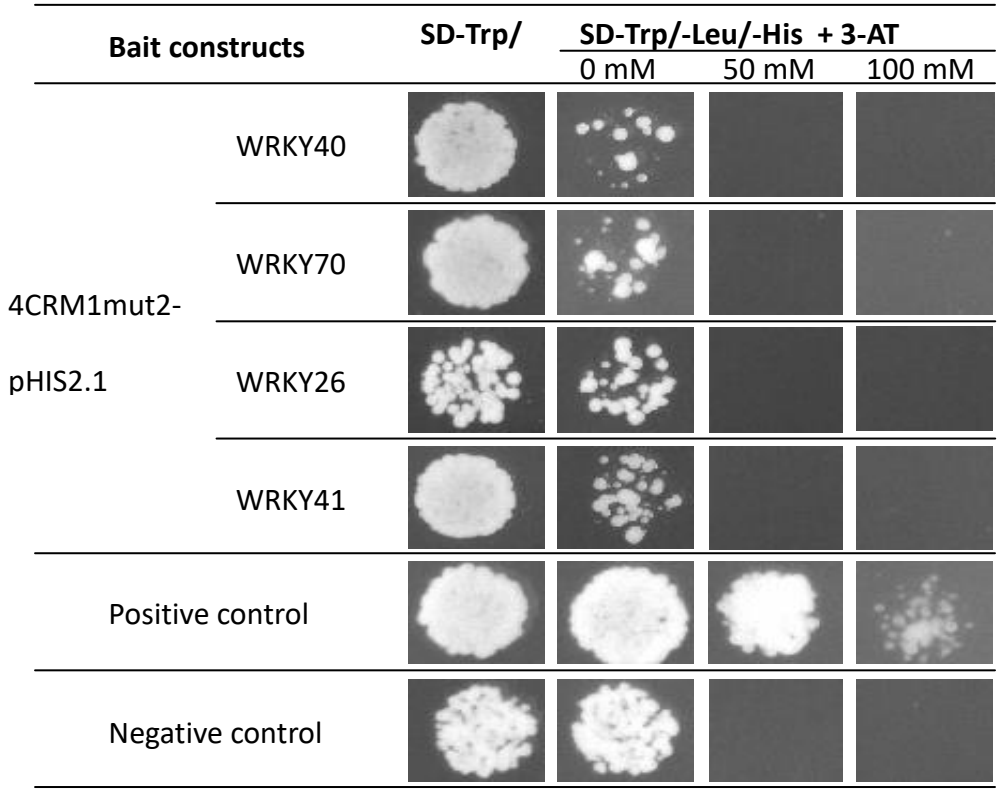
In addition to the whole CRM1 module, the function of its W-boxes and the WT-box which were shown to be important for MAMP responsivity (Figure 6) was also analyzed here. The prey vectors expressing WRKY26, 40, 41 and 70 were transformed into a bait strain containing a double mutation of the two W-boxes (4CRM1mu2) or a single mutation in the WT-box (4CRM1mu5) and the same assay for yeast expression was done as for the unmutated 4CRM1 construct. The results are shown in Figure 11B and 11C. No yeast growth was seen on 50 mM 3-AT medium when the bait construct harbours mutations in both W-boxes (4CRM1mut2, Figure 11B). In case of a mutation in the WT-box, it is obvious that WRKY40 and WRKY70 were positively selected in the presence of 50mM 3AT while WRKY26 and WRKY41 were not (4CRMmut5, Figure 11C). These results indicate the importance of the two W-boxes of CRM1 for interaction with all tested WRKY factors because yeast growth was completely inhibited in the presence of both mutated W-boxes. Furthermore, differential participation of the WT-box in interaction with WRKYs is suggested. First, the WT-box is not necessary for WRKY40 and WRKY70 because a mutation in the WT-box (4CRM1mut5) showed no effects in the presence of 50 mM 3AT. Second, in case of WRKY26 and WRKY41, it seems to be that the WT-box of CRM1 is required for MAMP responsivity as there were no signs of yeast growth when the WT-box was mutated. However, the presence of the WT-box itself is not sufficient for a binding

complex with WRKY26/WRKY41 which needs to be tight enough for gene activation. In summary, these results indicate that the binding of WRKY26 and WRKY41 needs both W-boxes and the WT-box of CRM1 while WRKY40 and WRKY70 require only the W-boxes.

(A)



(B)



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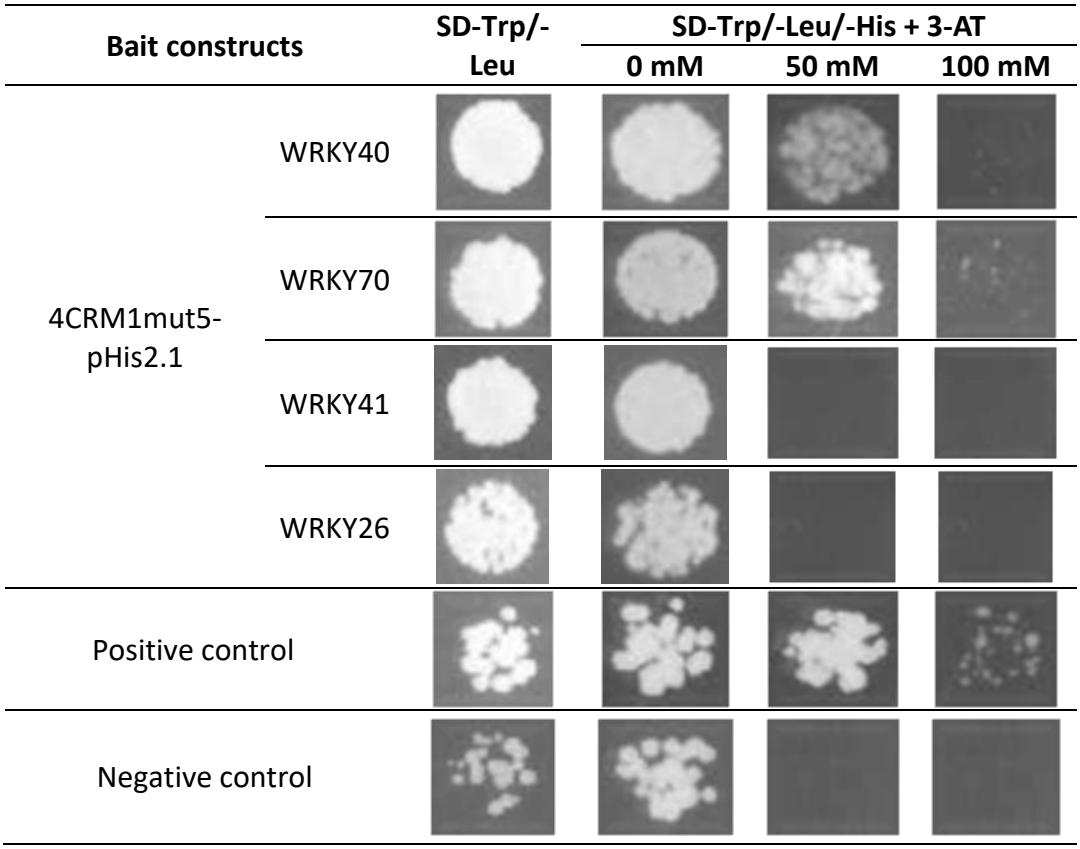


Figure 11. Selected WRKY factors require the W-boxes for reporter gene activation in yeast.

Prey plasmids coding for WRKY factors were retransformed into bait-containing yeast cells containing the unmutated CRM1 (A); the CRM1 in which all W-boxed were mutated, 4CRM1mut2 (B) and mutated WT-box of CRM1, 4CRM1mut5 (C). Transformed yeast were selected on triple drop out media (-Leu/-Trip/-His) harbouring 0, 50, and 100 mM 3-AT, respectively.

4.6. WRKY40, 41 and 70 act antagonistically on the CRM1 to repress or activate reporter gene activity

In this part, the interaction of CRM1 with WRKY26, 40, 41, and 70 that were described above is analyzed in the protoplast system. A plasmid expressing individual WRKYs was co-transformed with the plasmid containing tetramers of CRM1 into parsley protoplasts and the GUS reporter gene activity was measured after normalization with LUC activity.

WRKY40 was previously shown to be a repressor for gene expression (Shang et al., 2010) while WRKY41 was shown to act either as a repressor or an activator (Higashi et al., 2008). When we transformed only these WRKYs and the CRM1 expressing constructs into parsley protoplasts, no reporter gene induction was observed (Figure 12A and B, 4CRM1 + WRKY40 – Pep25 and 4CRM1 + WRKY41 – Pep25). Therefore it was necessary to use Pep25 as an inducer in our system. Figure 12 shows that parsley protoplasts cotransformed with the reporter vector (4CRM1) and a vector not expressing WRKY40 or WRKY41 (pORE) are strongly Pep25-responsive. When parsley cells are cotransformed with the reporter plasmid (4CRM1) and a plasmid expressing WRKY40 or WRKY41 also Pep25-responsive reporter gene expression is observed (Figure 12A and B). Comparing to the parsley protoplasts that is only transformed with CRM1 (pORE), a strong decrease of GUS activity was seen when WRKY40 or WRKY41 was co-transformed, meaning that both of these WRKYs are repressors of Pep25-responsive gene expression through CRM1 interaction (Figure 12A and B, compare 4CRM1 + pORE + Pep25 with 4CRM1 + WRKY40, 4CRM1 + WRKY41 – Pep25).

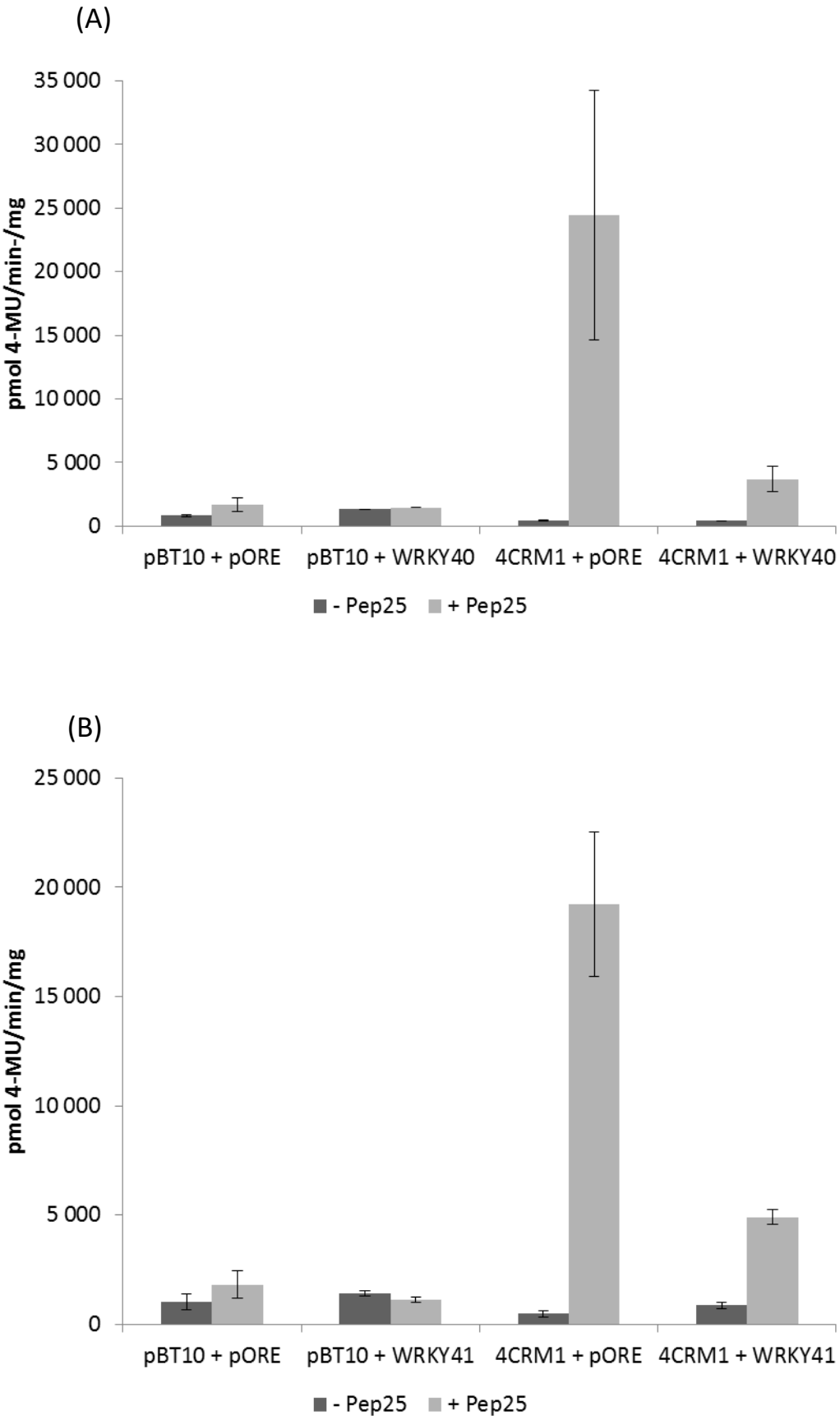


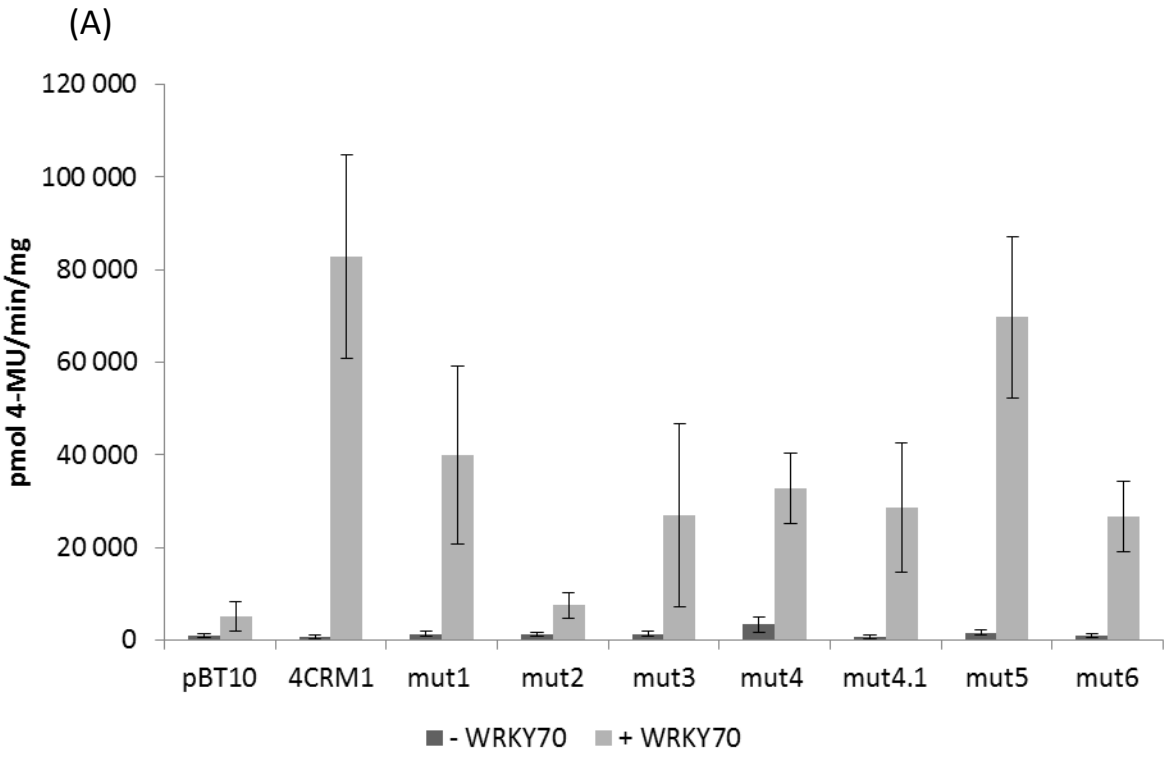
Figure 12. WRKY40 and 41 repress MAMP-responsive reporter gene expression

Transient reporter gene assays in parsley protoplasts after co-transformation of plasmids harbouring CRM1 with a WRK40 (A) and WRKY41 (B) expressing or a non-expressing plasmid

(pORE) in the presence and absence of Pep25. The empty vector pBT10GUS-d35SLUC (pBT10) serves as a negative control. The number of experiments was 2 (Refer to Table 10 and 11, Appendix).

In contrast, WRKY70 was shown to be an activator of gene expression (Machens et al., 2014). It was also nicely repeated in my data that the presence of WRKY70 strongly induced reporter gene activity when 4CRM1 was co-transformed with the WRKY70 expressing plasmid into parsley protoplasts (Figure 13). To analyze which part of CRM1 is more crucial for interaction with WRKY70, different mutations in CRM1 (mut1, mut2, mut3, mut4, mut4.1, mut5 and mut6) were tested in presence of WRKY70. The double mutation of the two W-boxes (mut2) had the strongest negative effect on GUS activity compared to all other mutations, showing the importance of the W-boxes. In contrast, mutation in the WT-box (mut5) showed almost no reduction of GUS activity compared to the wildtype 4CRM1 and to the other mutations. This data shows that the WT-box alone is not sufficient to induce the expression of the reporter gene in the presence of WRKY70 (mut2). Furthermore, the differential contribution of two W-boxes was analysed. A double mutation in the first W-box and the WT-box (mut3) led to a stronger decrease of GUS-activity comparing to the double mutation in the second W-box and the WT-box (mut1). In addition, single mutation in the first W-box (mut6) had comparable effect as the double mutation (mut3). In contrast, nearly no further decrease of GUS activity was seen when the second W-box was mutated (compare the double mutation mut3 with single mutations mut4 and mut4.1, Figure 13). In summary, these results indicate the requirement of the two W-boxes in CRM1, of which the first W-box is the more important one. In contrast, the WT-box does not seem to be necessary for gene induction through an interaction with the activator WRKY70.

We did the same experiment with WRKY26 but the results were unclear, thus we could not conclude whether this factor is a repressor or an activator for gene induction through binding with CRM1 (data not shown).



(B)

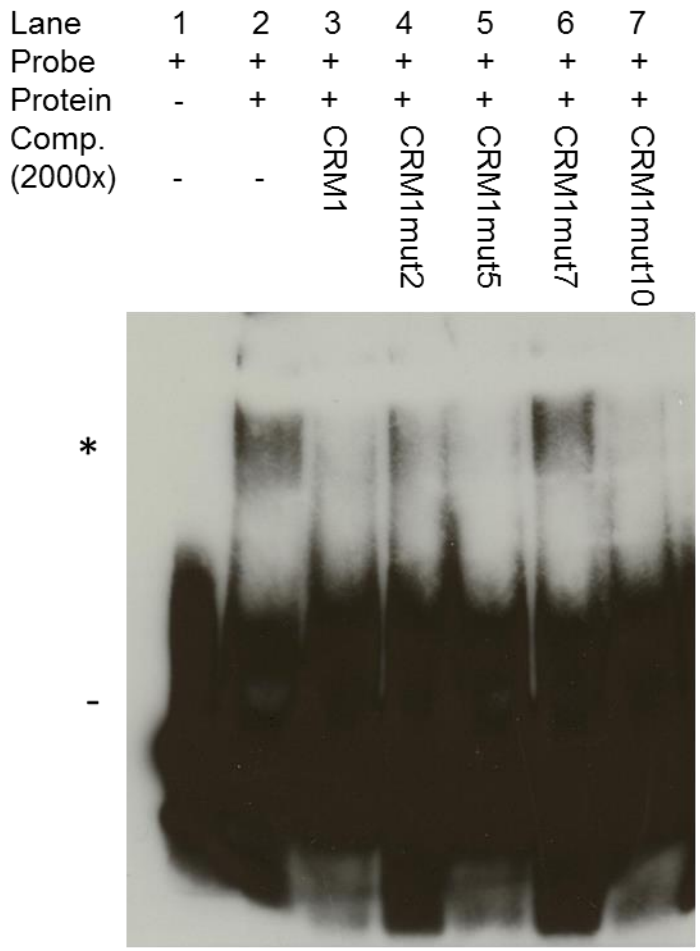
```
CRM1     TGGTCAGCATGTTGGACTTTCCAAATTCATTGACC
mut1     ----------AAGTCCCTT-------CCAGTT
mut2     -AACTG----------------------CCAGTT
mut3     -AACTG-------AAGTCCCTT-------------
mut4     ----------------------------CCAGTT
mut4.1   -----------------------------CAGT-
mut5     ----------AAGTCCCTT-------------
mut6     -AACTG-----------------------------
```

Figure 13. WRKY70 activates CRM1 driven reporter gene expression and requires the W-boxes for activation.

(A) Transient reporter gene assays in parsley protoplasts after co-transformation of plasmids harbouring CRM1 and selected mutations with and without a WRK70 expressing plasmid. The empty vector pBT10GUS-d35SLUC (pBT10) serves as a negative control. Normalized GUS values were obtained after transformation from 12 (pBT10 and 4CRM1 -WRKY70 and +WRKY70) and 6 (mut1, mut2, mut3, mut4, mut4.1, mut5, mut6 -WRKY70 and +WRKY70) independent experiments. In each experiment GUS expression was measured twice. The mean values and standard deviations were determined from all measurements (Refer to Table 9, Appendix). (B) The sequence of the CRM1 and that of seven mutations. Altered nucleotides in the sequences are shown, unaltered nucleotides are not shown (-). The two W-boxes and the WT-box are underlined.

4.7 WRKY70 binds to the two W-boxes of CRM1

The transactivation assays in parsley protoplasts and the interaction assay in yeast indicate that the W-boxes (first W-box GGTCA and second W-box TTGATC) of CRM1 can bind directly to WRKY70 and are essential for WRKY70-stimulated gene expression (Figures 11 and 13). In the following part I want to analyse this proposal and determine if the W-boxes are responsible for this interaction. For this, recombinant protein WRKY70 was expressed in *E. coli,* purified, and an electrophoretic mobility shift assay (EMSA) was performed. The results demonstrate that W-boxes of CRM1 are required for binding of WRKY70 *in vitro* (Figure 14). When the labeled probe of CRM1 was incubated with protein WRKY70, slower migrating complexes can be detected in comparison with the probe without recombinant protein (Figure 14, lanes 1 and 2). The slowest migrating DNA–protein complexes show the interaction of the full size recombinant protein WRKY70 with CRM1. These complexes are not detectable when an unlabelled CRM1 is added as a competitor, indicating that WRKY70 binds directly to CRM1 (Figure 14, lane 3). The same competition was also seen when the mutated WT-box (mut5) or mutated adjacent nucleotides (mut10) were used as a competitor, showing that the WT-box (mut5) and region between WT-box and W-box (mut10) are not necessary for CRM1 interaction with WRKY70 (Figure 14, lane 5 and 7). In contrast, the mutations in two W-boxes of the CRM1 (mutations 2) or a triple mutation (mut7) did not compete for WRKY70 binding when used as unlabelled competitors. This indicates that the nucleotides at the site of mutations are required for binding of WRKY70 (Figure 14, lanes 4 and 6). In summary the results indicate that the W-boxes of CRM1 can be bound specifically by WRKY70.




```
CRM1     TGGTCAGCATGTTGGACTTTCCAAATTCATTGACC
mut2     -AACTG----------------------CCAGTT
mut5     ----------AAGTCCCTT----------
mut7     -AACTG-------AAGTCCCTT-------CCAGTT
mut10    ------ATGCACC---------GGGCCTG------
```

Figure 14. Gel shift experiments with WRKY70 and CRM1 with different competitor sequences.

The CRM1 used as a probe and four mutations within this CRM1 used as competitor (Comp.) sequences are shown. Altered nucleotides in the CRM1 are indicated, unaltered nucleotides are not shown (−). Lane 1, free probe. Lane 2, free probe plus WRKY70. Lane 3, free probe plus WRKY70 and unlabelled probe in 2000-fold molar excess. Lane 4 through 7, free probe plus WRKY70 and unlabelled mutations of the probe as indicated in 2000-fold molar excess. Specific retarded DNA–protein complex was marked by open asterisks at the left-hand side of the figure, whereas a black bar designates the position of the free running probes.

5. Discussion

5.1 MAMP-responsive gene expression requires high organization of multiple components in the *WRKY30* promoter

Transcriptional reprogramming of cellular genes is considered a vital part of the plant kingdom when subjected to biotic or abiotic stresses. Under specific conditions, different regulatory gene families are induced and activate an appropriate defense response (Atkinson et al., 2013; Banerjee and Roychoudhury, 2015; Chen, L. et al., 2012). The *WRKY* family present in various plant species was shown to be strongly regulated during stress conditions (Pandey and Somssich, 2009; Rushton et al., 1996; Rushton et al., 2010). A lot of studies also demonstrate the importance of the interaction between W-box-containing promoters and WRKY transcription factors for responses of plants to pathogen-associated-, developmental or environmental changes (Cai et al., 2008; Liu et al., 2016; Rocher et al., 2005; Turck et al., 2004; Willmott et al., 1998; Yamamoto et al., 2004; Yu et al., 2001). In *Arabidopsis thaliana*, 74 WRKY proteins were identified based on available genomic and cDNA sequence data (Dong et al., 2003; Eulgem et al., 2000; Eulgem and Somssich, 2007; Yu et al., 2001).

A combination of bioinformatics and experimental approaches allowed the identification of conserved sequence motifs from 510 upregulated gene groups after fungal/oomycete exposure which were classified into 37 motif families with known and novel *cis*-sequences (Koschmann et al., 2012). Among these motifs, motif 27 contains sequences that harbor WRKY binding site similarities and are elicitor (Pep25)-responsive. The two sequence modules, CRM1 and CRM2, in the promoter region of transcription factor WRKY30 in *A. thaliana*, belong to motif 27 (Koschmann et al., 2012; Machens et al., 2014). In addition to the mentioned W-boxes (with the core sequence TGAC), these modules contain a novel core sequence (GACTTT) (Koschmann et al., 2012) (see Figure 5). It was recently shown that sequences C/TGACTTTT (designated as WT-box) are specifically bound by WRKY70 (Machens et al., 2014).

In order to better understand the functional roles of the W- and WT-boxes in the CRM1 and CRM2 from the *WRKY30* promoter, synthetic promoters harbouring mutations in these boxes were first tested in parsley protoplasts. For CRM1, a single mutation in the

first W-box almost abolished MAMP activity (mut6, Figure 6) compared to other single mutations, indicating the importance of this element. The single mutation in the WT-box also resulted in reduction of MAMP activity while the second W-box seems to have no effect (Figure 6). Similar results were obtained for these mutations when the study was expanded to *Arabidopsis thaliana* (mut4LUC, mut5LUC and mut6LUC, Figure 10). In parsley, the second W-box could restore some MAMP activity when the first W-box was mutated (compare single mut5 and double mut1, Figure 6) indicating that, in the co-presence of the other *cis*-elements, the second W-box could exhibit its function. In addition, the results from double mutations showed that the presence of the first W-box alone did not lead to detectable gene induction by Pep25 (mut1, Figure 6). Together with other double mutations (mut2 and mut3, Figure 6), these data indicate the synergy of W-boxes and WT-box in CRM1 for a full MAMP effect. Likewise, mutation analysis in CRM2 showed the importance of coordination between these *cis*-elements (mut2, mut3 and mut4, Figure 7) for Pep25 responsitivity.

In *A. thaliana*, 49 members of the WRKY superfamily were strongly regulated after *Pseudomonas syringae* or salicylic acid (SA) induction and 80% of them contain at least four W-boxes in their promoter regions (Dong et al., 2003). In another study, a highly significant enrichment of W-boxes were found in all of 26 promoters of *Arabidopsis* pathogenesis-related (*PR*) marker genes that are specifically bound by WRKY proteins under different systemic acquired resistance (SAR)- induced or repressed treatments (Maleck et al., 2000). Moreover, by using the Gene Ontology (GO) annotation at TAIR (http://www.arabidopsis.org/portals/genAnnotation/functional_annotation/go.jsp), Ciolkowski *et al*. found that genes harbouring the consensus TWGTTGACYWWWW in their 600 bp promoter are significantly enriched for proteins involved in stress responses or metabolism (Ciolkowski et al., 2008). From these studies, it seems to be that the frequent repetition of W-box clusters in these promoters may infer to their roles in gene regulations. Indeed, my results provide evidence for this structure-function inference of two W-boxes in both CRM1 and CRM2 (mut1, Figure 6; and mut2, Figure 7). Furthermore, my data reveals the importance of the WT-box which is consistent with a recent report (Lehmeyer et al., 2016). In that study, Lehmeyer and colleagues analysed the role of a tripartite 35-bp regulatory module in the promoter of *AtDJ1E*, a homologue of human

oncogene *DJ1*, which is induced by a variety of biotrophs and necrotrophs (Bülow et al., 2007; Zimmermann et al., 2004). All three *cis*-elements (GGACTTTT, GGACTTG and GCCACC) of this CRM are required for MAMP responsivity. The authors also revealed that the first WT-box is essential for SA-, ABA-, MeJA- and MeJA/ET-induced reporter gene activity while the second WT-box and GCC-box are contributory for those responsivity.

The conclusion of combinatorial effects between *cis*-elements in the *AtDJ1E* promoter (Lehmeyer et al., 2016) as well as in the *AtWRKY30* promoter (my study) is still of high value when we extent this view to other plant species. For example, in parsley, a strong effect of PcWRKY1 on transcription regulation requires more than two W-boxes (Eulgem et al., 1999). Likewise, the HvWRKY38 of barley needs two closely-located W-boxes for full activity (Marè et al., 2004). Moreover, the presence of W-box and non W-box (PRE2 and PRE4) in Os*Wrky13* also contribute to the induction of this gene in rice plants (Cai et al., 2008). In summary, our results are in line with previous studies showing the correlation of at least two *cis*-elements with full MAMP responsitivity.

In addition to W- and WT-boxes, adjacent nucleotides are demonstrated in my study to have at least some effects on MAMP activity. In parsley protoplasts, only the mutated sequence between the first W-box and the WT-box showed a reduction of the reporter gene activity (mut8, Figure 6) while the other sequence had no effects (mut9, Figure 6). However, it was interesting to see the decrease of the reporter gene activity of both mutated sequences in the *Arabidopsis* protoplasts (Figure 9). Perhaps the discrepancy is due to different networks of regulatory proteins in parsley and *Arabidopsis thaliana* that either bind to these sequences or not. Another reason might be the different characteristics of pathogen elicitors (Pep25 in Parsley and flg22 or AtPep1 in *A. thaliana*) that may trigger different MAMP-responsive pathways. Nevertheless, these results emphasize the contribution of adjacent nucleotides to the W- and WT-boxes in *WRKY30* to pathogen responses that was similar to the interaction of parsley *PR-1* promoter components with five different members of the WRKY family (Ciolkowski et al., 2008). Mutation of the G residue directly 5’ upstream of the W-box had a clearly negative effect on DNA binding of WRKY6 and WRKY11 but a strongly positive effect on WRKY26, WRKY38 and WRKY43. Another example for the function of neighbouring nucleotides of the W-box

is the drastic decrease of GUS-reporter activity when those nucleotides in WRKY18 promoter were mutated (Chen, C. and Chen, 2002).

In conclusion, my study could show the necessity of combinations between different *cis*-elements for effective MAMP-responsive gene activity. In addition to the classical W-box, the contribution of the newly identified WT-box, which will be discussed in more detail in chapter 5.3, was demonstrated in this study to be important for transcriptional regulation as well. Studies in future should focus on the contribution of these elements in the whole plant context, for example testing mutant lines of *A. thaliana* under pathogen induced conditions.

5.2 The diversity of WRKY factors interacting with CRM1 and CRM2 of the *WRKY30* promoter

WRKY30 was shown to be regulated under drought and salt conditions (Scarpeci et al., 2013). However, my preliminary tests did not confirm the reporter gene activation by CRM1 and CRM2 of the *WRKY30* promoter under these abiotic treatments in transgenic plants (data not shown). Thus my study focused on understanding the role of *WRKY30* promoter under biotic elicitors. As there is growing evidence of the interaction between WRKY proteins to form functional complexes in stress responses (Chi et al., 2013), it is interesting to see which WRKY factors interact with CRM1 and CRM2 of the *WRKY30* promoter.

Since both CRM1 and CRM2 of the investigated promoter contain the core DNA binding sites for WRKYs (Eulgem et al., 2000), it was not surprising for us that WRKY proteins were isolated from yeast one-hybrid TF screenings (Table 2). However, despite the fact of the same W-box core sequence (TTGAC/T), different WRKY factors were identified to target either CRM1 or CRM2. The discrepancy may be related either to the presence of different WT-boxes (GGACTTTC in CRM1 and TGACTTTT in CRM2) or to the different adjacent nucleotide sequences of CRM1 and CRM2. Until now, it was shown that WRKY70 could bind directly to the CGACTTTT/TGACTTTT sequence of WT-box (Machens et al., 2014, see Table 3) but no TFs were identified to interact with the GGACTTTT/GGACTTTG WT-box (Lehmeyer et al., 2016, see Table 3). Moreover, in spite of the fact that *AtWRKY30* promoter was shown in this study to be MAMP-responsive, no R protein-WRKYs including

AtWRKY16, AtWRKY19, and AtWRKY52 (Rinerson et al., 2015), were selected in our TF-screening. R protein-WRKY genes exist in flowering plants and they contain domains typical for both resistance (R) proteins and WRKY transcription factors. Perhaps these R protein-WRKYs do not directly target WRKY30, at least in our tested condition.

Besides, CRM1 specifically selected WRKY70 6 times while CRM2 was not targeted by this TF (Table 3). Also, our promoter deletion study showed that CRM2 is not as important for strong induction of the reporter genes as CRM1 (S5, Figure 8). Previously, CRM2 (seq22) was shown to strongly compete for WRKY70 binding to seq20 whereas seq24 (CRM1) is less efficient (Machens et al., 2014). One reason for this contradiction might be the difference in the used approaches. While Machens *et al*. (2014) tested the binding activity of seq22 (CRM2) and seq24 (CRM1) with WRKYs *in vitro*, I tested these activity in parsley protoplasts and could further demonstrate the importance of seq24 (CRM1) in yeast one-hybrid screens.

Li and colleagues showed that 3 WRKY factors including WRKY53, 54 and 70 could interact independently with WRKY30 (Li, J., 2014). Moreover, WRKY54 was classified into the same subgroup IIIb with WRKY70 (Kalde et al., 2003). However, only WRKY53 and WRKY70 were selected under our condition while WRKY54 was not selected (Table 2). On the other hand, Miao *et al* showed that WRKY30 is not a target of WRKY53 in their experimental system (Miao et al., 2004). Moreover, in our study WRKY53 did not reproducibly activate reporter gene in yeast cells (Figure 11A). This contradiction might be due to a dual function of WRKY53 (Kalde et al., 2003; Miao et al., 2004). In addition, other TFs might interact with WRKY53 to make a triple complex that repress or activate WRKY30 depending on the specific third TF. One of those TF could be WRKY41 as the encoding gene *WRKY41* was co-induced with *WRKY53* in suspension-cultured cells and in leaves of *A. thaliana* by flagellin treatment (Higashi et al., 2008). Also both, WRKY41 and WRKY53, were found to be selected in our TF screening. An example for the proposed TF complex is the interaction between WRKY18, WRKY40 and WRKY60 that leads to interesting functions of these TFs upon fungal treatments (Chen, H. et al., 2010).

From all selected WRKY factors with CRM1, only WRKY26, 40, 41, and 70 were confirmed to drive reporter gene activity in yeast (Figure 11A). Yeast experiments using a double

mutation in the W-boxes (mut2, Figure 11B) show the importance of the W-boxes for all of these WRKYs. Furthermore, the similar experiments with a single mutation in the WT-box (mut5, Figure 11C) show the differential requirement of the WT-box between WRKY factors (see a detailed discussion in chapter 5.3).

WRKY26 was selected twice with CRM1 and once with CRM2 in yeast one-hybrid screenings. The selection with CRM2 is not expected because both W-boxes have the sequence TTGACT which does not bind to WRKY26 *in vitro* (Ciolkowski et al., 2008). Thus, this selection is currently unclear which might also explain the unclear function of WRKY26 in plant cells (see section 4.6).

WRKY40 and WRKY41 were shown in this study to repress CRM1 of the *WRKY30* promoter in its MAMP response (Figure 12, section 4.6). The conclusion of WRKY40 as a repressor is in line with the previous study in *Arabidopsis* under abscisic acid and abiotic stresses (Chen, H. et al., 2010). Meanwhile, WRKY41 was shown previously to be either a repressor or an activator under different treatments (Ding et al., 2014; Higashi et al., 2008); especially, the function of WRKY41 relates to the three adjacent W-boxes in ABA-induced promoters (Ding et al., 2014). Thus, in our study, the presence of the middle WT-box in CRM1 may also contribute to the repressive role of WRKY41 to the target WRKY30. Our results added two more members (WRKY40 and WRKY41) to the previously described repressor TF groups including WRKY18, WRKY25 and WRKY48 (Chen, C. and Chen, 2002; Xing et al., 2008; Zheng et al., 2007). For example, the overexpression of WRKY18 showed a stunt growth of the mutant plant while a transgenic line expressing a moderate level of this TF revealed an enhancement of pathogenesis-related genes after *Pseudomonas syringae* infection (Chen, C. and Chen, 2002).

Among all WRKYs that gave positive signals in the yeast assay, WRKY70 is the only activator for the CRM1 of WRKY30. Together with (Besseau et al., 2012) and (Machens et al., 2014), this study could expand the list of target promoters for WRKY70 (Li, J. et al., 2004). Moreover, WRKY70 was also shown to be positive regulators of plant disease resistance (AbuQamar et al., 2006; Li, J. et al., 2006). A transgenic *WRKY70 A. thaliana* line was prepared for further study the role of WRKY30-WRKY70 interaction in the whole plant. Moreover, the function of this complex can be further tested in *Nicotiana tabacum*, a popular plant model.

5.3 The WT-box of the *AtWRKY30* promoter is a novel *cis*-element in MAMP responsivity

In addition to the classical W-box, in this thesis, I could also confirm the recent observation on the function of the newly designated WT-box (Machens et al., 2014) and provide further insights into its participation for regulation of the *WRKY30* gene. In line with Machens *et al*., I saw the contribution of WT-box in both CRM1 (mut5, Figure 6) and CRM2 (mut4 and mut5, Figure 7). Thus these results expand the current information about non-W-boxes of *WRKY* promoters (Breeze et al., 2011; Cai et al., 2008; Lehmeyer et al., 2016; Sun, 2003). However, in case of CRM2, it is not clear whether the single mutations (mut4 and mut5, Figure 7) affect the WT-box only or both, the WT-box and adjacent nucleotides between two W-boxes. Perhaps future studies should deepen this analysis.

Furthermore, the function of the WT-box of CRM1 was analysed in yeast using a bait construct harboring a single mutation in the WT-box (section 4.5). This WT-box is not necessary for the binding of WRKY40 and WRKY70, whereas it is necessary for WRKY26 and WRKY41 in the reporter gene expression assay. On one hand, these four WRKY factors share the same conserved WRKY domain (WRKYGQK) which is the classical binding site to the W-box (Ülker and Somssich, 2004); therefore this explains why they require two W-boxes. On the other hand, the differentiation of interaction between these TFs with the WT-box still needs to be investigated. One reason could be the nucleotides adjacent to the core sequence GACTTT of the WT-box. Machens *et al*. showed that WRKY70 could bind specifically to the sequence CGACTTTT or TGACTTTT (Machens et al., 2014) whereas no TFs have been found until now to interact with the sequence GGACTTT(T/G) (Lehmeyer et al., 2016). The results from the Machens and the Lehmeyer study fit well with my data as there was no effect on yeast growth when the mutated GGACTTTC construct was co-transformed with WRKY70. This means WRKY70 interact with the W-boxes of CRM1 but not with the WT-box. This was also confirmed by EMSA analysis (section 4.7). WRKY40 might interact only with the W-boxes of CRM1 in a similar way as WRKY70 but it works as a repressor (chapter 5.2). From the case of WRKY26 and WRKY41 which seems to interact with the sequence GGACTTTC of the *WRKY30* promoter (Table 3), it is obvious that this WT-box is functionally important for MAMP responsivity. This observation is in line with previous studies showing the requirement of non-W-box in response of plants to biotic or

abiotic stress (Bolivar et al., 2014; Scarpeci et al., 2008). Moreover, it is noteworthy that this WT-box of the CRM1-WRKY30 (GGACTTTC) is similar to the nuclear factor κB (NF-κB) binding site (Verma et al., 1995). It is also highly similar to the NF-κB binding site GGGACTTTCC in the enhancer of the immunoglobulin light chain gene (Ig-B) gene (Escalante et al., 2002). These similarities might relate to the contribution of the WT-box in pathogen response but need further studies to confirm.

Higashi and colleagues showed that WRKY41 is a key regulator of *PR5* and *PDF1.*2 expression in response to flagellin but the specific interaction domains of this TF have not yet been determined in that context (Higashi et al., 2008). Likewise, WRKY26 was shown to cooperate with WRKY25 and WRKY33 in mediating plant thermotolerance (Li, S. et al., 2011). It was proved to interact with the W-box of parsley *PR1* promoter region (Ciolkowski et al., 2008). But no information about its interaction with non-W box is available. Therefore, similar to the case of WRKY41, our result is the first demonstration revealing the WT-box of *AtWRKY30* as target of WRKY26 in yeast one-hybrid screening. It would be interesting in future studies to see the transcriptional outcomes of this interaction in *A. thaliana* in pathogen response.

The amino acid sequence of WRKY proteins is one crucial factor determining their interaction with target genes. For instance, Ciolkowski showed that amino acid exchanges in various region of the WRKY11 protein strongly alter DNA binding capacity to the *AtSIRKpW11* promoter region. In that study, not only the WRKY domain but also the adjacent amino acids at the N'terminus as well as in the zinc-finger domain at the C'terminus have profound effects on DNA binding ability of the protein (Ciolkowski et al., 2008). Therefore, in addition to check the presence of a typical WRKY domain as mentioned above, I also analysed the amino acid composition of all four WRKY factors in order to understand the discrimination in selection by the CRM1 (Figure 15). Although WRKY70 and WRKY41 belong to group III with a distinct zinc-finger-like motif (C_2-HC) (Kalde et al., 2003), they show different interaction with the WT-box. Thus a specific interaction between that zinc-finger domain with the WT-box could be excluded. It is also right for the case of WRKY26 (group I) and WRKY40 (group IIa); both of which contain the unique zinc-finger motif (C_2-H_2) (Eulgem et al., 2000) but interact differently with the WT-box of CRM1. However, concerning the adjacent amino acids of the consensus WRKY

domain, it would be an explanation for the diversity of these TF in interaction with the WT-box. Directly preceding the WRKYGQK stretch is Asn (N) and Arg (R) in WRKY26, a Gln (Q) in WRKY40 and a Ser (S) in WRKY41/70 (Figure 15). Despite having the same S at the upstream WRKY region, the downstream amino acid is different between WRKY41 (Asp(D)) and WRKY70 (Glu (E)) (aligment of AtWRKY domains using *A. thaliana* WRKY protein database, http://www.ncbi.nlm.nih.gov/; Figure 15). Furthermore, WRKY26 contains two WRKY domains which might also affect the binding activity of this TF to different *cis*-elements. To understand whether these sequence differences of the four TFs are related to the various WT-box interactions, mutation analyses of those WRKYs need to be studied.

In summary, the presence of the WT-box in the *AtWRKY30* promoter emphasizes the contribution of a non-classical element in regulating MAMP-responsivity. Perhaps a larger TF-screening library would allow to isolate more specific TFs interacting with this WT-box and to figure out the function of this *cis*-element for plant cells in pathogen defense.

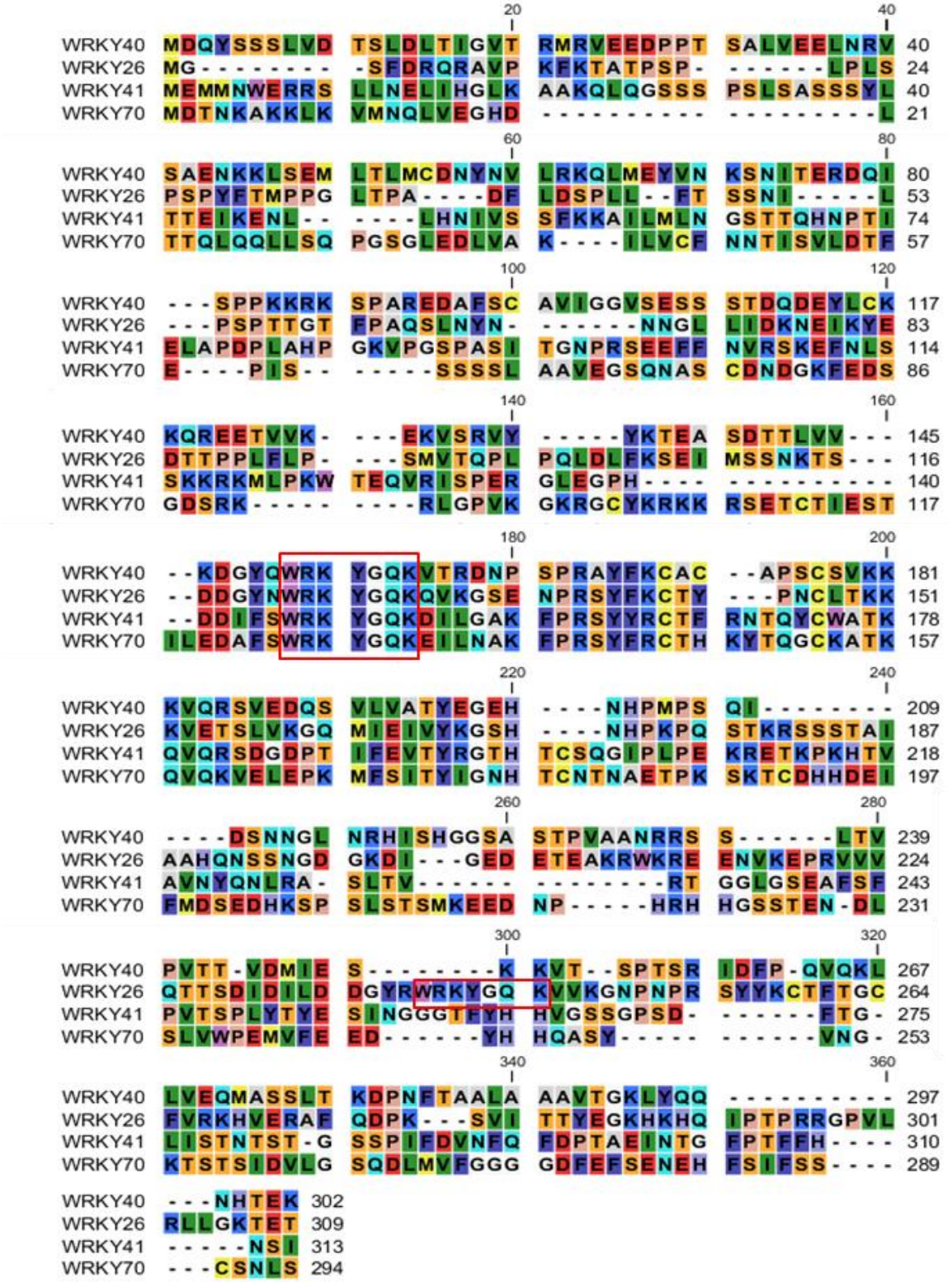


Figure 15. Alignment of amino acid sequences of four WRKY factors that showed interaction with CRM1 of WRKY30 and were able to drive reporter gene activity in yeast.

The amino acid sequences at the N´terminus and at the C´terminus of each WRKY are downloaded from NCBI and aligned by CLC Workbench software. Gaps (dots) have been inserted for optimal

alignment. Red boxes indicate conserved WRKYGQK regions of WRKY transcription factors. Please note that WRKY26 contains two WRKY domains.

Table 3. Two types of WT-boxes involved in MAMP-responsive gene expression

WT-box	Class	Binding TF	Reference
CGACTTTT	I	WRKY70	(Machens et al., 2014)
TGACTTTT	I	WRKY70	
GGACTTTT	II	n.d.	(Lehmeyer et al., 2016)
GGACTTTG	II	n.d.	
GGACTTTC	II	WRKY26 and WRKY41*	this study

*The interaction was only shown in yeast cells

6. Summary

Plants have various mechanisms to develop their innate immunity in response to pathogen attacks. Transcription factors (TF) play important roles in regulating essential pathways for the protection of plants. In light of recent advances in the combination of bioinformatic and experimental approaches, a diversity of DNA binding motifs for TFs were discovered in genes strongly upregulated by microbe-associated molecular patterns (MAMPs). Amongst these 25 elicitor-responsive sequences, two *cis*-regulatory modules (CRM1 and CRM2) from the *Arabidopsis thaliana WRKY30* promoter were previously demonstrated to interact with WRKY70, one member of the largest family of transcriptional regulators in plants. Both modules harbour two W-boxes (TGACC; TTGACC in CRM1 and TTGACT; TTGACT in CRM2) and one WT-box (GGACTTTC in CRM1 and TGACTTTT in CRM2).

To understand the role of these modules in the MAMP response in detail, a synthetic promoter system combined with a mutation approach was used in my study. Deletion analysis of the *WRKY30* promoter in parsley protoplasts shows that CRM1 is important for MAMP responsivity but CRM2 alone is not sufficient. This result is consistent with data in *Arabidopsis thaliana* protoplats where only CRM1 shows flg22 and AtPep1 responsivity.

Mutation analysis of the synthetic promoters harbouring CRM1 or CRM2 reveals that the W- and WT-boxes are necessary for MAMP-responsive gene expression in parsley protoplasts, of which one W-box is more important than the other. The results show that at least two regulatory sites are required for full MAMP responsivity in the CRM1. Further mutation analysis in *Arabidopsis* protoplasts shows the requirement of the two W-boxes and the WT-box of the CRM1 for flg22 and AtPep1 responsivity.

Yeast one-hybrid screenings using both CRMs with a 1,500 transcription factors (TFs) only prey library select mainly WRKY-factors. While CRM2 randomly selects a wide array of different WRKYs, CRM1 predominantly selects WRKY26, 40, 41 and 70 by their interaction with the W-boxes in CRM1.

In plant cells WRKY40 and 41 act as repressors of CRM1-responsive gene expression while WRKY70 is an activator. In agreement with previous studies, my study shows that in yeast, all of four investigated WRKY factors could bind to the classical W-boxes of CRM1. However, WRKY40 and WRKY70 do not bind to the WT-box whereas WRKY26 and WRKY41 seem to interact with this element of CRM1. These results demonstrate the importance of

the WT-box of CRM1 as a new *cis*-regulatory sequence for MAMP-induced gene expression requiring at least a second *cis*-regulatory sequence for MAMP responsivity.

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Appendix

Table 4. Normalized GUS values of the CRM1 (seq24) analysis of *WRKY30* gene (pmol 4-MU/min/mg)

Constructs	Experiments	-Pep25	Average	Standard deviation	+Pep25	Average	Standard deviation	Significance
pBT10	1	623.835	756.006	281.123	1078.692	815.549	216.544	0.715
		489.710			674.894			
	2	606.077			555.106			
		527.303			789.245			
	3	1077.207			662.833			
		1211.905			1132.526			
4D	1	2176.889	5755.863	4568.873	36520.661	44607.697	12451.241	0.000
		2980.062			42523.420			
	2	3688.862			38152.587			
		4402.556			36710.802			
	3	5600.917			69131.018			
		15685.895						
S24	1	303.353	2016.649	2699.166	14716.114	20905.657	8744.196	0.000
		269.189			12071.822			
	2	420.196			9886.004			
		507.033			14171.535			
	3	778.305			34395.226			
		9593.133			34395.226			
	4	3460.013			20691.036			
		2204.131			24677.733			
	5	1328.205			14928.054			
		1302.933			29123.814			
S24mut1	1	691.650	695.913	11.526	1202.020	986.715	179.703	0.031
		681.030			826.190			
	2	712.850			1125.980			
		698.120			792.670			
S24mut2	1	913.150	806.613	70.776	654.180	811.240	136.707	0.960
		713.950			914.100			
	2	801.080			700.430			
		798.270			976.250			
S24mut3	1	1056.950	1061.468	31.590	961.180	1037.730	58.879	0.561
		1074.270			1089.590			
	2	1013.890			1000.010			
		1100.760			1100.140			
S24mut4	1	1060.047	1787.153	675.130	26548.039	28590.031	7033.083	0.000
		1052.389			32546.784			
	2	1649.039			19736.347			
		1669.206			27315.962			

	3	2926.838			23662.637			
		2365.399			41730.417			
S24mut4.1	1	587.620	677.937	104.749	16552.749	15585.806	4623.348	0.001
		567.368			22352.212			
	2	737.577			9612.020			
		819.181			13826.245			
S24mut5	1	771.031	1034.794	254.751	7697.985	5992.919	1524.984	0.000
		847.439			5877.617			
	2	931.736			4917.337			
		1107.494			3309.993			
	3	999.036			6673.426			
		1552.027			7481.157			
S24mut6	1	463.598	607.035	160.057	740.314	1281.768	403.147	0.006
		388.862			1784.116			
	2	530.834			1321.136			
		786.429			793.175			
	3	652.113			1338.670			
		820.377			1713.197			
S24mut7	1	1042.011	864.380	177.631	661.002	518.277	142.725	0.007
		686.750			375.552			
	2	1042.011			661.002			
		686.750			375.552			
	3	1042.011			661.002			
		686.750			375.552			
S24mut8	1	567.570	706.498	126.913	10777.809	9245.090	4010.701	0.010
		596.285			8644.857			
	2	799.886			3247.183			
		862.249			14310.511			
S24mut9	1	2483.010	2259.867	283.158	21049.735	22896.398	4107.098	0.000
		2595.521			19098.433			
	2	1947.834			21613.385			
		2013.103			29824.038			

Table 5. Normalized GUS values of the CRM2 (seq22) analysis of *WRKY30* gene (pmol 4-MU/min/mg)

Constructs	Experiments	-Pep25	Average	Standard deviation	+Pep25	Average	Standard deviation	Significance
pBT10	1	1277.738	1687.664	1260.883	1044.362	2750.842	2625.037	0.350
		829.345			987.106			
	2	467.090			440.289			

		163.561			227.821			
	3	3051.234			5311.676			
		4144.500			6967.393			
	4	1992.822			5953.030			
		1575.027			1075.056			
4D	1	5628.052	7509.184	2172.131	43488.260	52047.558	14806.512	0.000
		5088.454			68992.876			
	2	9327.687			43143.657			
		9992.544			68571.195			
					45203.792			
					23618.031			
					62157.176			
					61205.478			
S22	1	1124.655	2703.387	2239.613	11173.089	17471.058	5769.675	0.000
		1861.452			18857.737			
	2	1780.221			24836.906			
		1570.542			7965.535			
	3	1163.126			18305.829			
		1163.126			19200.969			
	4	5618.951			13953.882			
		7345.021			25474.514			
Mut1	1	2571.155	2553.141	692.326	6904.249	6682.207	1144.628	0.002
		3664.938			7438.894			
	2	1842.285			7631.569			
		2134.183			4754.118			
Mut2	1	743.124	669.744	84.646	2210.425	1773.821	420.014	0.004
		665.943			1631.446			
	2	737.032			2101.821			
		532.875			1151.593			
Mut3	1	799.706	1228.520	319.401	978.204	1507.328	542.264	0.273
		1110.252			1145.675			
	2	1676.060			1523.360			
		511.789			2382.073			
Mut4	1	1013.874	571.767	260.886	1027.513	1464.345	1081.646	0.237
		390.166			413.186			
	2	511.789						
		371.238			2952.335			
Mut5	1	2892.340	1999.192	587.349	2442.222	1984.639	578.646	0.977
		1255.667			991.993			
	2	1829.042			2279.086			
		2019.718			2225.256			

Table 6. Normalized GUS values of the native promoter analysis of *WRKY30* gene (pmol 4-MU/min/mg)

Constructs	Experiments	-Pep25	Average	Standard deviation	+Pep25	Average	Standard deviation	Significance
pBT10	1	826.214	1011.471	201.532	814.545	1287.201	379.725	0.112
		634.598			606.929			
	2	1205.047			1852.265			
		1009.745			1322.805			
	3	1242.620			1292.465			
		965.461			1558.065			
	4	1242.620			1292.465			
		965.461			1558.065			
4D	1	5982.171	4822.116	2930.095	75849.572	68099.790	8071.681	0.000
		8452.001			70184.189			
	2	3570.528			53470.581			
		8173.776			74730.887			
	3	1853.943			66263.718			
		900.279			66263.718			
S1	1	592.252	786.254	147.749	6679.479	9822.410	2627.588	0.087
		663.832			7479.479			
	2	1070.387			14305.995			
		928.411			10003.233			
	3	788.603			10355.969			
		649.717			90155.969			
	4	831.058			8767.374			
		765.775			7767.374			
S2	1	711.241	938.428	205.599	3257.754	4353.756	1146.515	0.000
		891.636			3264.653			
	2	1110.322			6132.505			
		1302.835			6132.644			
	3	1058.270			3349.402			
		849.918			4595.491			
	4	959.995			3525.799			
		623.207			4571.804			
S3	1	916.545	1036.562	393.201	3044.817	3780.717	1090.252	0.000
		841.053			2198.622			
	2	1749.533			2540.493			
		1610.399			3880.493			
	3	821.458			5089.506			
		689.983			2719.764			
	4	612.289			4272.827			
		1051.238			5258.990			
S4	1	1733.376	1914.082	199.902	1638.434	1786.323	182.157	0.445
		2181.747			1578.097			
	2	2031.478			1919.186			

		1709.724			2009.577			
S5	1	1144.768	1568.821	245.135	1163.621	1561.790	326.064	0.977
		1701.803			1460.983			
	2	1730.144			2068.262			
		1698.568			1554.294			

Table 7. Normalized LUC values of the mutation of CRM1 (seq24) analysis of *WRKY30* gene (pmol LUC/GUS) with flg22

Constructs	Experiments	-Pep25	Average	Standard deviation	+Pep25	Average	Standard deviation	Significance
pBT10	1	1.564	2.887	1.249	0.820	3.376	2.489	0.771
	2	1.942			1.141			
	3	3.307			4.825			
	4	4.735			6.717			
S24	1	2916.590	2818.816	1489.555	9402.474	10559.088	4129.217	0.022
	2	838.027			4718.458			
	3	2501.526			12024.202			
	4	5019.120			16091.218			
Mut4	1	358.943	212.565	117.798	821.046	1051.277	453.597	0.021
	2	97.850			733.557			
	3	95.693			815.878			
	4	297.773			1834.627			
Mut5	1	13.039	7.799	4.305	25.017	65.527	36.767	0.036
	2	5.353			62.312			
	3	2.112			49.973			
	4	10.690			124.804			
Mut6	1	31.714	20.031	9.967	168.372	221.334	45.713	0.000
	2	12.962			244.640			
	3	7.719			188.356			
	4	27.731			283.967			
Mut8	1	324.851	208.360	77.012	2956.105	3695.397	1626.299	0.010
	2	229.873			2882.796			
	3	130.211			6492.276			
	4	148.504			2450.411			
Mut9	1	420.907	552.148	320.272	1843.499	2314.595	943.149	0.022
	2	234.480			1109.861			
	3	467.246			2662.302			
	4	1085.957			3642.716			

Table 8. Normalized LUC values of the mutation of CRM1 (seq24) analysis of *WRKY30* gene (pmol LUC/GUS) with AtPep1

Constructs	Experiments	-AtPep1	Average	Standard deviation	+AtPep1	Average	Standard deviation	Significance
pBT10	1	4.457	3.320	0.896	2.018	2.224	1.109	0.338
	2	2.268			3.674			
	3	3.235			0.980			
S24	1	1382.294	1426.063	714.757	9009.855	8516.139	4111.118	0.037
	2	2322.521			13286.165			
	3	573.373			3252.398			
Mut4	1	73.134	140.442	90.247	536.334	1002.449	949.643	0.270
	2	268.006			2326.280			
	3	80.186			144.734			
Mut5	1	3.267	3.625	1.212	15.192	19.740	10.423	0.096
	2	5.255			34.157			
	3	2.352			9.871			
Mut6	1	5.392	10.458	6.913	42.261	104.659	83.229	0.186
	2	20.232			222.290			
	3	5.750			49.426			
Mut8	1	49.909	149.372	133.323	824.909	1784.130	1554.309	0.212
	2	337.823			3976.556			
	3	60.385			550.927			
Mut9	1	128.940	368.352	312.977	882.898	1828.770	1362.683	0.214
	2	810.461			3755.788			
	3	165.654			847.624			

Table 9. Normalized GUS values of the mutation of CRM1 (seq24) analysis with WRKY70 (pmol 4-MU/min/mg)

Constructs	Experiments	pORE	Average	Standard deviation	WRKY70-pORE	Average	Standard deviation	Significance
pBT10G/L	1	2026.316	1193.904	446.676	8132.753	6199.885	2934.539	0.000
		1464.550			7103.184			
	2	2120.463			6902.524			
		1824.839			8300.114			
	3	1100.570			5678.021			
		1104.224			6664.513			
	4	1056.437			3493.772			
		853.494			5243.697			
	5	1109.686			9727.516			
		1871.262			12780.915			
	6	830.898			5978.032			
		647.288			7443.275			
	7	863.973			978.320			
		819.532			965.823			

	8	494.364			707.713			
		688.194			741.832			
	9	1105.768			8210.172			
		1102.843			6350.942			
	10	1094.684			6296.462			
		788.738			6847.380			
	11	1854.023			6742.937			
		1410.674			7252.968			
	12	1051.940			6861.278			
		1368.931			9393.091			
Seq24	1	1203.614	783.015	288.428	51802.964	76777.063	30320.626	0.000
		1106.885			128117.957			
	2	1108.425			40324.399			
		781.166			74420.568			
	3	644.919			46338.693			
		836.337			30949.384			
	4	626.930			32740.416			
		610.470			39641.771			
	5	1003.419			119284.212			
		628.919			122247.626			
	6	675.996			100435.359			
		433.186			69474.946			
	7	505.653			105760.054			
		245.448			104133.977			
	8	410.463			62851.905			
		345.790			65267.514			
	9	971.479			100265.548			
		562.557			44060.428			
	10	806.954			51840.049			
		733.297			92799.766			
	11	1182.456			79675.587			
		1281.786			106737.737			
	12	1038.849						
		1047.358			96701.600			
Seq24mut1	1	2105.230	1347.859	551.731	52476.229	39963.899	19124.776	0.000
		1841.613			62744.499			
	2	1858.787			25849.448			
		2351.503			23695.738			
	3	1478.862			24381.628			
		1087.689			19809.492			
	4	1391.434			20302.713			
		838.070			23674.668			
	5	881.166						
		927.261			70138.418			
	6	743.253			59420.412			
		669.442			57109.641			
Seq24mut2	1	1961.218	1228.205	447.846	9029.289	7589.059	2777.665	0.000
		1193.748						
	2	2095.598			8516.962			
		1851.829			10243.318			
	3	1246.982			4422.006			
		904.628			8370.973			

	4	873.159			3037.866			
		914.659			2244.938			
	5	1041.607			9320.754			
		921.962			9320.754			
	6	805.795			10429.093			
		927.278	1360.006	479.941	8543.696			
Seq24mut3	1	2115.924			26935.797	26930.375	19717.820	0.000
		1813.835			17842.373			
	2	2186.876			16634.269			
		1327.673			8075.870			
	3	1369.218			10708.201			
		1664.137			16973.062			
	4	1248.001			5457.526			
		1067.340			7439.592			
	5	1148.642			61101.987			
		1027.190			56900.759			
	6	773.078			46528.540			
		578.157			48566.525			
Seq24mut4		1400.946	3357.156	1629.801	52582.090	32758.943	7590.290	0.000
	1	1374.473			39820.760			
		1110.215			33379.463			
	2	1046.031			26031.822			
		5046.602			26218.598			
	3	3373.325			39131.433			
		3399.667			27776.380			
	4	3834.309			26072.412			
		5628.545			27606.797			
	5	4957.310			31806.216			
		4410.260			28300.222			
	6	4704.189			34381.127			
		367.613	724.615	302.928	38388.748	28657.955	13986.998	0.000
Seq24mut4.1	1	523.301			59450.079			
		255.166			32425.823			
	2	324.112			38738.443			
		827.739			30600.474			
	3	827.151			19412.123			
		688.714			13323.786			
	4	619.666			14362.979			
		900.157			14614.266			
	5	1093.524			18864.204			
		1042.803			45022.018			
	6	1225.433			18692.514			
		1272.648	1585.525	525.155	95186.965	69735.790	17481.868	0.000
Seq24mut5	1	908.301			95960.928			
		816.440			62809.355			
	2	655.688			65710.476			
		1578.345			55522.521			
	3	1913.889			95326.674			
		1991.579			54662.899			
	4	1873.300			87006.583			
		1873.100			61964.988			
	5	2370.104			54860.791			

		1710.530			61073.121			
	6	2062.375			46744.179			
Seq24mut6	1	778.151	1020.855	335.995	41786.363	26662.357	7656.686	0.000
		622.979			34862.422			
	2	467.661			18255.002			
		464.235			21677.317			
	3	1514.929			28649.220			
		1094.237			27563.718			
	4	1155.844			20271.972			
		1348.825			29491.788			
	5	1209.525			17482.244			
		1241.180			18214.513			
	6	1259.654			25027.763			
		1093.042			36665.958			

Table 10. Normalized GUS values of the mutation of CRM1 (seq24) analysis with WRKY40 (pmol 4-MU/min/mg)

Constructs	Experiments	-Pep25	Average	Standard deviation	+Pep25	Average	Standard deviation	Significance
pBT10	1	757.409	833.152	75.743	1149.951	1684.812	534.861	0.256
	2	908.895			2219.673			
pBT10-WRKY40	1	1306.432	1319.405	12.973	1462.864	1446.384	16.480	0.026
	2	1332.378			1429.904			
4S24-pORE	1	409.304	432.285	22.981	34240.777	24420.341	9820.436	0.135
	2	455.266			14599.905			
4S24-WRKY40	1	377.311	399.181	21.869	4659.729	3679.240	980.489	0.079
	2	421.050			2698.751			

Table 11. Normalized GUS values of the mutation of CRM1 (seq24) analysis with WRKY41 (pmol 4-MU/min/mg)

Constructs	Experiments	-Pep25	Average	Standard deviation	+Pep25	Average	Standard deviation	Significance
pBT10	1	1368.367	1021.090	347.277	2451.909	1816.386	635.524	0.387
	2	673.813			1180.862			
pBT10-WRKY41	1	1270.418	1409.067	138.649	1020.186	1127.723	107.538	0.250
	2	1547.716			1235.261			
4S24-pORE	1	627.894	468.210	159.684	22512.875	19221.618	3291.257	0.030
	2	308.526			15930.361			
4S24-WRKY41	1	724.884	858.524	133.640	5231.685	4907.477	324.208	0.007
	2	992.163			4583.269			

Table 12. List of oligos and primers

Number	Name of sequences	Sequences
7056	S24_mut1f	ctagtTGGTCAGCATGTTAAGTCCCTTAAATTCACCAGTTt
7057	S24_mut1r	ctagaAACTGGTGAATTTAAGGGACTTAACATGCTGACCAa
7058	S24_mut2f	ctagtTAACTGGCATGTTGGACTTTCCAAATTCACCAGTTt
7059	S24_mut2r	ctagaAACTGGTGAATTTGGAAAGTCCAACATGCCAGTTAa
7060	S24_mut3f	ctagtTAACTGGCATGTTAAGTCCCTTAAATTCATTGACCt
7061	S24_mut3r	ctagaGGTCAATGAATTTAAGGGACTTAACATGCCAGTTAa
7062	S24_mut4f	ctagtTGGTCAGCATGTTGGACTTTCCAAATTCACCAGTTt
7063	S24_mut4r	ctagaAACTGGTGAATTTGGAAAGTCCAACATGCTGACCAa
7064	S24_mut5f	ctagtTGGTCAGCATGTTAAGTCCCTTAAATTCATTGACCt
7065	S24_mut5r	ctagaGGTCAATGAATTTAAGGGACTTAACATGCTGACCAa
7066	S24_mut6f	ctagtTAACTGGCATGTTGGACTTTCCAAATTCATTGACCt
7067	S24_mut6r	ctagaGGTCAATGAATTTGGAAAGTCCAACATGCCAGTTAa
7068	S24_mut7f	ctagtTAACTGGCATGTTAAGTCCCTTAAATTCACCAGTTt
7069	S24_mut7r	ctagaAACTGGTGAATTTAAGGGACTTAACATGCCAGTTAa
8108	Sq24mut4.1f	5‘ ctagtTGGTCAGCATGTTGGACTTTCCAAATTCATCAGTCt 3‘
8109	Sq24mut4.1r	5’ ctagaGACTGATGAATTTGGAAAGTCCAACATGCTGACCAa 3‘
7091	AtWRKY40 F	ggatccaCATGGATCAGTACTCATCCT *BamH*I
7092	AtWRKY40 R	ggtaccGAATGTATTGGAGATTGT *Kpn*I
7093	AtWRKY41 F	gagctcaaATGGAAATGATGAATTGGGAGC *Sac* I
7094	AtWRKY41 R	ggtaccGTACTACTTAAATCGAATTGTGG *Kpn*I
7095	AtWRKY26 F	ggatccCATTGATGGGCTCTTTTGATCG *BamH*I
7096	AtWRKY26 R	ggtaccGGCGCATGATTAAGGAAACA *Kpn*I

7097	MS23 GUS-LUC_f	GGAAAACTCGACGCAAGAAA
7098	MS23 GUS-LUC_r	GGTTTCTACAGGACGGACCA
7214	W26_seqf:	GGCGCAAATACGGGCAGAAGC
7215	W26_seqr:	CAC TCT TGG CTC CTT CAC ATT
7216	W40_seqf	TTCAGCTGCGCGGTTATTGGC
7217	W40_seqr	CGATCTGCGATGGCATTGGAT
7218	W41_seqf	GTAAGATCGAAAGAATTCAAC
7219	W41_seqr	GTAATTTACTGCTACTGTGTG
7220	S22mut1f	CTAGTTCGTTCTTCGACTGGAAAGTCAAACTATCTCTCTCT
7221	S22mut1r	CTAGAGAGAGAGATAGTTTGACTTTCCAGTCGAAGAACGAA
2722	pQE_F	CGGATAACAATTTCACACAG
7223	pQE_pro_F	CCCGAAAAGTGCCACCTG
7224	pQE_R	GTTCTGAGGTCATTACTGG
7225	pHis_left	TGGCAAGTGTAGCGGTCA
7226	pHis_rigt	TCGTTTATCTTGCCTGCTCAT
7227	W41F_SacI	GAGCTCATGGAAATGATGAATTGGGAGC
7228	Seq22mut4_f	5´ctagtTCGTTCTTCAGTCGGGGGACTAAACTATCTCTCTCt 3´
7229	Seq22mut4_r	5´ctagaGAGAGAGATAGTTTAGTCCCCCGACTGAAGAACGAa 3‘
7230	Seq22mut5_f	ctagtTCGTTCTTCAGTCAAGGAGTCAAACTATCTCTCTCt
7231	Seq22mut5_r	ctagaGAGAGAGATAGTTTGACTCCTTGACTGAAGAACGAa
7232	Seq24mut8_f	ctagtTGGTCAATGCACCGGACTTTCCAAATTCATTGACCt
7233	Seq24mut8_r	ctagaGGTCAATGAATTTGGAAAGTCCGGTGCATTGACCAa
7234	Seq24mut9_f	ctagtTGGTCAGCATGTTGGACTTTCCGGGCCTGTTGACCt
7235	Seq24mut9_r	ctagaGGTCAACAGGCCCGGAAAGTCCAACATGCTGACCAa
7236	Seq24mut10_f	ctagtTGGTCAATGCACCGGACTTTCCGGGCCTGTTGACCt

7237	Seq24mut10_r	ctagaGGTCAACAGGCCCGGAAAGTCCGGTGCATTGACCAa
7238	S1_SpeIF_230bp	GCactagtTGGTCAGCATGTTGGACTTTC
7239	S2_SpeIF_217bp	GCactagtGGACTTTCCAAATTCATTGACC
7240	S3_SpeIF_201bp	GCactagtTTGACCAAAGACTGGTCTCAC
7241	S4_SpeIF_155bp	GCactagtTCGTTCTTCAGTCAAAAAGTC
7242	S5_SpeIF_120bp	GCactagtACACATCCTCTTTAAATTCTCC
7243	S_pro_XhoIR	CGctcgagTACGTTCAAAGAGTGGAG
7244	M13 Reverse	CAGGAAACAGCTATGAC
7245	M13 Forward	GTAAAACGACGGCCAG
7246	GUS75	GCGATCCAGACTGAATGCC
7247	Cmyc_F:	AGCCTCGACCTCAACACAAC
7248	WRKY70_cmyc_755_R	CGAACCATGATGACGATGAG
7249	4xcis_seq f	GCGCGGTGTCATCTATGTTA from ≠6825
7250	4xcis_seq r	GAGCGTGTCCTCTCCAAATG from ≠6826
7251	pBT10LUC_1792 f	GGCCTTTTGCTCACATGTTC
7252	pBT10LUC_2284 r	ATTCCGCGTACGTGATGTTC
7253	WRKY70_319bp_f	GAACCCATCTCCTCCTCCTC
7254	WRKY70_319bp_r	GCTCAACCTTCTGGACTTGC
7255	Cmyc_213bp_F	GTCTAGAAGGCCTTGGATCC
7256	Cmyc_213bp_R	GAGGAGGAGGAGATGGGTTC
7257	4WT-boxS24_F	ctagtGGACTTTCCtctagtGGACTTTCCtctagtGGACTTTCCtctagtGGACTTTCCt
7258	4WT-boxS24_R	ctagaGGAAAGTCCactagaGGAAAGTCCactagaGGAAAGTCCactagaGGAAAGTCCa

7259	4W-boxS24_F	ctagtGGTCAtctagtGGTCAtctagtGGTCAtctagtGGTCAt
7260	4W-boxS24_R	ctagaTGACCactagaTGACCactagaTGACCactagaTGACCa
7209	LUC_xhoI_F	ctcgagTGGCCACCATGGAAG
7210	S1-230bp-SalI XbaI-R	gtcgactctagaTACGTTCAAAGAGTGGAG
7211	LUC-f1-R	CACCCTTAGGTAACCCAG
7212	LUC-f2-F	CATCTCATCTACCTCCCG
7213	LUC-f3-F	CCGCTGAATTGGAATCG
7261	S-pro-XbaI-R	CGtctagaTACGTTCAAAGAGTGGAG
7262	XhoI SpeI XbaI SalI_F	TCGAGACTAGTTCTAGAG
7263	XhoI SpeI XbaI SalI_R	TCGACTCTAGAACTAGTC
7268	S1_EcoRI_230bpF	GCgaattcTGGTCAGCATGTTGGACTTTC
7269	S2_EcoRI_217bpF	GCgaattcGGACTTTCCAAATTCATTGACC
7270	S3_EcoRI_201bpF	GCgaattcTTGACCAAAGACTGGTCTCAC
7271	S4_EcoRI_155bpF	GCgaattcTCGTTCTTCAGTCAAAAAGTC
7272	S5_EcoRI_120bpF	GCgaattcACACATCCTCTTTAAATTCTCC
7273	4WT-S24-F	aattcGGACTTTCCGGACTTTCCGGACTTTCCGGACTTTCCgagct
7274	4WT-S24-R	cGGAAAGTCCGGAAAGTCCGGAAAGTCCGGAAAGTCCg
7275	4WTS24-EcoRI-F	aattcGGACTTTCCGGACTTTCCGGACTTTCCGGACTTTCCa
7276	4WTS24-SpeI-R	ctagtGGAAAGTCCGGAAAGTCCGGAAAGTCCGGAAAGTCCg
6148	GAL4AD-RV	CGTTTTAAAACCTAAGAGTCAC
6149	GAL4AD	CTATTCGATGATGAAGATACCCC
7087	Prey_attB_fwd	AAGCAGGCTTCATG

6419	Bait_right GUSLUC	GCGAGCTCTCTTGCGGTCGACTCTA
6420	Bait_left GUSLUC	ATCGGGAATTAGATCTGTC
5121	pHis_left	TGGCAAGTGTAGCGGTCA
5122	pHis_right	TCGTTTATCTTGCCTGCTCAT
7568	S24_f	ctagtTGGTCAGCATGTTGGACTTTCCAAATTCATTGACCt
7569	S24_r	ctagaGGTCAATGAATTTGGAAAGTCCAACATGCTGACCAa

Table 13. List of strains maintained as glycerol stocks

Number	Name	Description
4534	4S24_pHis2.1	4xS24 (4×30H_8_M1S2)_pHis2.1 in *E. coli* XL1 blue, LB Kan^r
4535	4S24_pHis2.1 in Y1H-Gold	4S24_pHis2.1 in Y1H-Gold (Bait construct) , no growth SD-Trp, SD-Trp/His including 50 mM 3-AT.
4594	pHis2.1-sq24 + AT1G80840/WRKY40	pHis2.1-sq24(4×30H_8_M1S2) + AT1G80840/WRKY40 in Y1H-Gold, Medium SD-Trp/-Leu, Positive 3-AT
4595	pHis2.1-sq24 + At5g07100/WRKY26	pHis2.1-sq24(4×30H_8_M1S2) + At5g07100/WRKY26 in Y1H-Gold, Medium SD-Trp/-Leu, Positive 3-AT
4596	pHis2.1-sq24 + At1g69310/WRKY57	pHis2.1-sq24(4×30H_8_M1S2) + At1g69310/WRKY57 in Y1H-Gold, Medium SD-Trp/-Leu, Negative 3-AT
4597	pHis2.1-sq24 + At4g23810/WRKY53	pHis2.1-sq24(4×30H_8_M1S2) + At4g23810/WRKY53 in Y1H-Gold, Medium SD-Trp/-Leu, Negative 3-AT
4598	pHis2.1-sq24 + At3g56400/WRKY70	pHis2.1-sq24(4×30H_8_M1S2) + At3g56400/WRKY70 in Y1H-Gold, Medium SD-Trp/-Leu, Positive 3-AT
4599	pHis2.1-sq24 + AT4G11070/WRKY41	pHis2.1-sq24(4×30H_8_M1S2) + AT4G11070/WRKY41 in Y1H-Gold, Medium SD-Trp/-Leu, Positive 3-AT
4600	pHis2.1-sq24 + At4g31550/WRKY11	pHis2.1-sq24(4×30H_8_M1S2) + At4g31550/WRKY11 in Y1H-Gold, Medium SD-Trp/-Leu, Negative 3-AT
4601	S24_mut_1 monomer	pBT10GUSLUC_S24 _mut_1 (oligos #7056 and #7057) monomer, XL1 blue, LB Carb^r.
4602	S24_mut_2 monomer	pBT10GUSLUC_S24 _mut_2(oligos #7058 and #7059) monomer, XL1 blue, LB Carb^r.
4603	S24_mut_3	pBT10GUSLUC_S24 _mut_3 (oligos #7060 and #7061)

	monomer	monomer, XL1 blue, LB Carb^{r}.
4604	S24_mut_4 monomer	pBT10GUSLUC_S24 _mut_4 (oligos #7062 and #7063) monomer, XL1 blue, LB Carb^{r}.
4605	S24_mut_5 monomer	pBT10GUSLUC_S24 _mut_5 (oligos #7064 and #7065) monomer, XL1 blue, LB Carb^{r}.
4606	S24_mut_6 monomer	pBT10GUSLUC_S24 _mut_6 (oligos #7066 and #7067) monomer, XL1 blue, LB Carb^{r}.
4607	S24_mut_7 monomer	pBT10GUSLUC_S24 _mut_7 (oligos #7068 and #7069) monomer, XL1 blue, LB Carb^{r}.
4608	2xS24_mut_1	Dimer, pBT10GUSLUC_S24(30H_8_M1S2)_mut_1 (oligos #7056/57), XL1 blue, LB Carb^{r}.
4609	2xS24_mut_2	Dimer, pBT10GUSLUC_S24(30H_8_M1S2)_mut_2 (oligos #7058/59), XL1 blue, LB Carb^{r}.
4610	2xS24_mut_3	Dimer, pBT10GUSLUC_S24(30H_8_M1S2)_mut_3 (oligos #7060/61), XL1 blue, LB Carb^{r}.
4611	2xS24_mut_4	Dimer, pBT10GUSLUC_S24(30H_8_M1S2)_mut_4 (oligos #7062/63), XL1 blue, LB Carb^{r}.
4612	2xS24_mut_5	Dimer, pBT10GUSLUC_S24(30H_8_M1S2)_mut_5 (oligos #7064/65), XL1 blue, LB Carb^{r}.
4613	2xS24_mut_6	Dimer, pBT10GUSLUC_S24(30H_8_M1S2)_mut_6 (oligos #7066/67), XL1 blue, LB Carb^{r}.
4614	2xS24_mut_7	Dimer, pBT10GUSLUC_S24(30H_8_M1S2)_mut_7 (oligos #7068/69), XL1 blue, LB Carb^{r}.
4615	4xS24_mut_1	Tetramer, pBT10GUSLUC_S24(30H_8_M1S2)_mut_1 (oligos #7056/57), XL1 blue, LB Carb^{r}.
4616	4xS24_mut_2	Tetramer, pBT10GUSLUC_S24(30H_8_M1S2)_mut_2 (oligos #7058/59), XL1 blue, LB Carb^{r}.
4617	4xS24_mut_3	Tetramer, pBT10GUSLUC_S24(30H_8_M1S2)_mut_3 (oligos #7060/61), XL1 blue, LB Carb^{r}.
4618	4xS24_mut_4	Tetramer, pBT10GUSLUC_S24(30H_8_M1S2)_mut_4 (oligos #7062/63), XL1 blue, LB Carb^{r}.
4619	4xS24_mut_5	Tetramer, pBT10GUSLUC_S24(30H_8_M1S2)_mut_5 (oligos #7064/65), XL1 blue, LB Carb^{r}.
4620	4xS24_mut_6	Tetramer, pBT10GUSLUC_S24(30H_8_M1S2)_mut_6 (oligos #7066/67), XL1 blue, LB Carb^{r}.
4621	4xS24_mut_7	Tetramer, pBT10GUSLUC_S24(30H_8_M1S2)_mut_7 (oligos #7068/69), XL1 blue, LB Carb^{r}.
4648	WRKY26-pORE	WRKY26-pORE-d35SpA in *E. coli* XL1-blue LB Kan^{R} Linker

		*BamH*I and *Kpn*I
4649	WRKY40-pORE	WRKY40-pORE-d35SpA in *E. coli* XL1-blue LB Kan^{R} Linker *BamH*I and *Kpn*I
4650	WRKY41-pORE	WRKY41-pORE-d35SpA in *E. coli* XL1-blue LB Kan^{R} Linker *Sac*I and *Kpn*I
4651	Seq24mut4-pHis2.1-WRKY26	Seq24(4×30H_8_M1S2)mut4-pHis2.1- WRKY26 in Y1H-Gold, Medium SD-Trp/-Leu, Positive 3-AT
4652	Seq24mut4-pHis2.1-WRKY40	Seq24(4×30H_8_M1S2)mut4-pHis2.1- WRKY40 in Y1H-Gold, Medium SD-Trp/-Leu, Positive 3-AT
4653	Seq24mut4-pHis2.1-WRKY41	Seq24(4×30H_8_M1S2)mut4-pHis2.1- WRKY41 in Y1H-Gold, Medium SD-Trp/-Leu, Positive 3-AT
4654	Seq24mut4-pHis2.1-WRKY70	Seq24(4×30H_8_M1S2)mut4-pHis2.1- WRKY70 in Y1H-Gold, Medium SD-Trp/-Leu, Positive 3-AT
4655	Seq24mut5-pHis2.1-WRKY26	Seq24(4×30H_8_M1S2)mut5-pHis2.1- WRKY26 in Y1H-Gold, Medium SD-Trp/-Leu, Negative 3-AT
4656	Seq24mut5-pHis2.1-WRKY40	Seq24(4×30H_8_M1S2)mut5-pHis2.1- WRKY40 in Y1H-Gold, Medium SD-Trp/-Leu, Positive 3-AT
4657	Seq24mut5-pHis2.1-WRKY41	Seq24(4×30H_8_M1S2)mut5-pHis2.1- WRKY41 in Y1H-Gold, Medium SD-Trp/-Leu, Negative 3-AT
4658	Seq24mut5-pHis2.1-WRKY70	Seq24(4×30H_8_M1S2)mut5-pHis2.1- WRKY70 in Y1H-Gold, Medium SD-Trp/-Leu, Positive 3-AT
4659	Seq24mut6-pHis2.1-WRKY26	Seq24(4×30H_8_M1S2)mut6-pHis2.1- WRKY26 in Y1H-Gold, Medium SD-Trp/-Leu, Negative 3-AT
4660	Seq24mut6-pHis2.1-WRKY40	Seq24(4×30H_8_M1S2)mut6-pHis2.1- WRKY40 in Y1H-Gold, Medium SD-Trp/-Leu, Negative 3-AT
4661	Seq24mut6-pHis2.1-WRKY41	Seq24(4×30H_8_M1S2)mut6-pHis2.1- WRKY41 in Y1H-Gold, Medium SD-Trp/-Leu, Negative 3-AT
4662	Seq24mut6-pHis2.1-WRKY70	Seq24(4×30H_8_M1S2)mut6-pHis2.1- WRKY70 in Y1H-Gold, Medium SD-Trp/-Leu, Negative 3-AT
4663	4xSeq24mut4-pHis2.1 in Y1H-Gold	4xSeq24 (4x30H_8_M1S2)mut4-pHis2.1 in Y1H-Gold, SD-Trp
4664	4xSeq24mut5-pHis2.1 in Y1H-Gold	4xSeq24 (4x30H_8_M1S2)mut5-pHis2.1 in Y1H-Gold, SD-Trp
4665	4xSeq24mut6-pHis2.1 in Y1H-Gold	4xSeq24 (30H_8_M1S2)mut6-pHis2.1 in Y1H-Gold, SD-Trp
4666	1xSeq24mut4.1-pBT10GUSLUC	1xSeq24mut4.1-pBT10GUSLUC in *E. coli* XL1 blue, LB Carb^{r}
4669	2xSeq24mut4.1-	2xSeq24mut4.1-pBT10GUSLUC in *E. coli* XL1 blue, LB Carb^{r}

	pBT10GUSLUC	
4670	4xSeq24mut4.1-pBT10GUSLUC	4xSeq24mut4.1-pBT10GUSLUC in *E. coli* XL1 blue, LB Carb^{r}
4686	4xSeq24mut4.1-pHis2.1	4xSeq24mut4.1-pHis2.1 in *E. coli* XL1 blue,LB Kan^{R}
4693	4xSeq22mut2-pBT10GUSLUC	4xSeq22mut2-pBT10GUSLUC in *E. coli* XL1 blue, LB Carb^{r}
4694	4xSeq22mut3-pBT10GUSLUC	4xSeq22mut3-pBT10GUSLUC in *E. coli* XL1 blue, LB Carb^{r}
4724	W26_pQE32	WRKY26_pQE32 in XL1 Blue, LB Carb^{r}
4725	W40_pQE32	WRKY40_pQE32 in XL1 Blue, LB Carb^{r}
4726	W41_pQE32	WRKY41_pQE32 in XL1 Blue, LB Carb^{r}
4727	W26_pQE32	WRKY26_pQE32 in BL21 codon plus, LB Carb^{r}
4728	W40_pQE32	WRKY40_pQE32 in BL21 codon plus, LB Carb^{r}
4729	W41_pQE32	WRKY41_pQE32 in BL21 codon plus, LB Carb^{r}
4752	W26_pQE32	WRKY26_pQE32 in M15, LB Carb^{r}, Kan^{r}
4753	W40_pQE32	WRKY40_pQE32 in M15, LB Carb^{r}, Kan^{r}
4754	W41_pQE32	WRKY41_pQE32 in M15, LB Carb^{r}, Kan^{r}
4756	W26_pORE_cmyc	WRKY26_pORE_d35S_pA_cmyc in XL1-blue, LB kan^{r}
4757	W40_pORE_cmyc	WRKY40_pORE_d35S_pA_cmyc in XL1-blue, LB kan^{r}
4758	W41_pORE_cmyc	WRKY41_pORE_d35S_pA_cmyc in XL1-blue, LB kan^{r}
4761	W26_pORE-d35S-pA-cmyc (C58C1)	WRKY26_pORE-d35S-pA-cmyc in *A. tumefaciens* C58C1 strain. LB: Carb, Rifa, Kan 50 mg/L.
4762	W40_pORE-d35S-pA-cmyc (C58C1)	WRKY40_pORE-d35S-pA-cmyc in *A. tumefaciens* C58C1 strain. LB: Carb, Rifa, Kan 50 mg/L.
4763	W41_pORE-d35S-pA-cmyc (C58C1)	WRKY41_pORE-d35S-pA-cmyc in *A. tumefaciens* C58C1 strain. LB: Carb, Rifa, Kan 50 mg/L.
4764	W41F_SacI_pCR2.1	WRKY41F_SacI_pCR2.1 in E.*coli*, LB Carb^{r}
4765	1xseq22mut4	Monomer 1xseq22mut4_pBT10GUSLUC in *E.coli*, LB Carb^{r}
4767	2xseq22mut4	Dimer 2xseq22mut4_pBT10GUSLUC in *E.coli*, LB Carb^{r}
4768	4xseq22mut4	Tetramer 4xseq22mut4_pBT10GUSLUC in *E.coli*, LB Carb^{r} 4x(ctagtTCGTTCTTCAGTCGGGGGACTAAACTATCTCTCTCt)
4769	1xseq22mut5	Monomer 1xseq22mut5_pBT10GUSLUC in *E.coli*, LB Carb^{r}
4770	1xseq24mut8	Monomer 1xseq24mut8_pBT10GUSLUC in *E.coli*, LB Carb^{r}

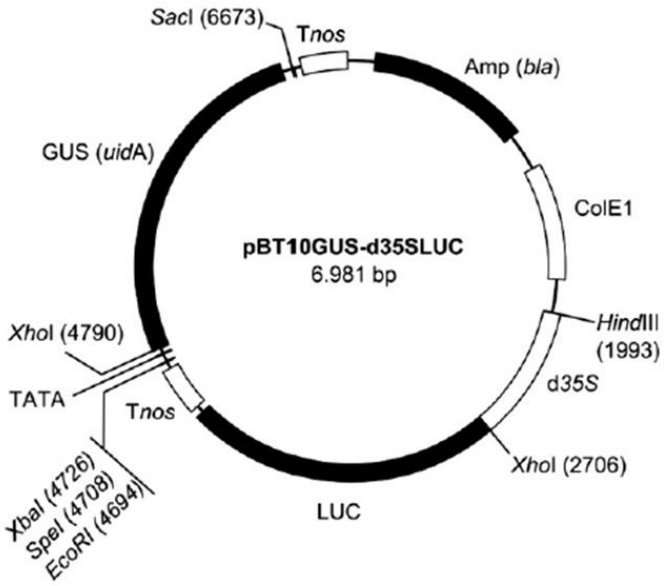
4771	1xseq24mut9	Monomer 1xseq24mut9_pBT10GUSLUC in *E.coli*, LB Carb^r
4772	1xseq24mut10	Monomer 1xseq24mut10_pBT10GUSLUC in *E.coli*, LB Carb^r
4773	2xseq22mut5	Dimer 2xseq22mut5_pBT10GUSLUC in XL1 bue, LB Carb^r
4774	S2-pBT10GUSLUC	217bp of the *WRKY30* promoter in pBT10GUSd35SLUC, in XL1 blue, LB Carb^r; linker SpeI and XhoI. Without TATA box
4775	S4-pBT10GUSLUC	155bp of the *WRKY30* promoter in pBT10GUSd35SLUC, in XL1 blue, LB Carb^r, linker SpeI and XbaI. Without TATA box
4776	S1-pBT10GUSLUC	230bp of the *WRKY30* promoter in pBT10GUSd35SLUC, in XL1 blue, LBcarb; linker SpeI and XhoI. Without TATA box
4777	4xseq22mut5	4xseq22mut5_pBT10GUSLUC in *E.coli*, LB Carb^r 4x(ctagtTCGTTCTTCAGTCAAGGAGTCAAACTATCTCTCTCt)
4778	2xseq24mut9	Dimer 2xseq24mut9_pBT10GUSLUC in *E.coli*, LB Carb^r
4779	4xseq24mut9	Tetramer 4xseq24mut9_pBT10GUSLUC in *E.coli*, LB Carb^r 4x(CTAGTTGGTCAGCATGTTGGACTTTCCGGGCCTGTTGACCT)
4780	2xseq24mut8	Dimer 2xseq24mut8_pBT10GUSLUC in *E.coli*, LB Carb^r
4781	2xseq24mut10	Dimer 2xseq24mut10_pBT10GUSLUC in *E.coli*, LB Carb^r
4782	4xseq24mut8	Tetramer 4xseq24mut8_pBT10GUSLUC in *E.coli*, LB Carb^r 4x(CTAGTTGGTCAATGCACCGGACTTTCCAAATTCATTGACCT)
4783	4xseq24mut10	Tetramer 4xseq24mut10_pBT10GUSLUC in *E.coli*, LB Carb^r 4x(CTAGTTGGTCAATGCACCGGACTTTCCGGGCCTGTTGACCT)
4793	S5_pCR2.1	120 bp of the *WRKY30* promoter in pCR2.1, XL1-blue, LB Carb^r, linker SpeI and XhoI.
4794	S1_pCR2.1	230 bp of the *WRKY30* promoter in pCR2.1, XL1-blue, LB Carb^r linker SpeI and XhoI.
4795	S2_pCR2.1	217 bp of the *WRKY30* promoter in pCR2.1, XL1-blue, LB Carb^r linker SpeI and XhoI.
4796	S3_pCR2.1	201 bp of the *WRKY30* promoter in pCR2.1, XL1-blue, LB Carb^r linker SpeI and XhoI.
4797	S4_pCR2.1	155 bp of the *WRKY30* promoter in pCR2.1, XL1-blue, LB Carb^r linker SpeI and XhoI.
4801	4xS24mut4_pBTLUC	Tetramer of seq24mut4 in pBTLUC vector, linker *EcoR*I and *Xba*I, *E.coli* XL1 blue, LB Carb^r.
4802	4xS24mut5_pBTLUC	Tetramer of seq24mut5 in pBTLUC vector, linker *EcoR*I and *Xba*I, *E.coli* XL1 blue, LB Carb^r.
4803	4xS24mut6_pBTLUC	Tetramer of seq24mut6 in pBTLUC vector, linker *EcoR*I and *Xba*I, *E.coli* XL1 blue, LB Carb^r.

4804	4xS24mut8_pBTLUC	Tetramer of seq24mut8 in pBTLUC vector, linker *EcoR*I and *Xba*I, *E.coli* XL1 blue, LB Carb^r.
4805	4xS24mut9_pBTLUC	Tetramer of seq24mut9 in pBTLUC vector, linker *EcoR*I and *Xba*I, *E.coli* XL1 blue, LB Carb^r.
4810	S3-pBT10GUSLUC	201 bp of the *WRKY30* promoter in pBT10GUSd35SLUC, in XL1 blue, LBcarb, linker SpeI/XhoI; Without TATA box
4828	S5-pBT10GUSLUC	120 bp of the *WRKY30* promoter in pBT10GUSLUC, XL1 blue, LB Carb^r linker SpeI and XbaI. Without TATA box
4829	pBT10GUS-d35SLUC_D	Vector for cloning native promoter
4830	S1-pBTLUC	230 bp of the *WRKY30* promoter in pBTLUC, in XL1 blue, LBcarb; linker SpeI/XhoI; without TATA box
4831	S2-pBTLUC	217 bp of the *WRKY30* promoter in pBTLUC, in XL1 blue, LB Carb^r; linker SpeI/XhoI; without TATA box
4832	S3-pBTLUC	201 bp of the *WRKY30* promoter in pBTLUC, in XL1 blue, LBcarb; linker SpeI/XhoI; without TATA box
4833	S4-pBTLUC	155 bp of the *WRKY30* promoter in pBTLUC, in XL1 blue, LB Carb^r; linker SpeI/XhoI; without TATA box
4834	S5-pBTLUC	120 bp of the *WRKY30* promoter in pBTLUC, XL1-blue, LBcarb ; linker SpeI/XhoI; without TATA box
4835	4WTSeq24_pHis2.1	4xWT-box of Seq24, linker EcoRI/SpeI aattcGGACTTTCCGGACTTTCCGGACTTTCCGGACTTTCCa in pHis2.1; XL1 blue, Kan^r
4863	4WTSeq24_pHis2.1 in Y1H-Gold	4xWT-box of Seq24 in pHis2.1, linker EcoRI/SpeI, in Y1H-Gold, SD medium –Trp.
4864	4S24mut2_pHis2.1	4xseq24mut2, linker EcoRI/SacI 4ctagtTAACTGGCATGTTGGACTTTCCAAATTCACCAGTTt in pHis2.1; XL1 blue, Kan^r
4865	4S24mut2_pHis2.1 in in Y1H-Gold	4xseq24mut2, linker EcoRI/SacI, 4ctagtTAACTGGCATGTTGGACTTTCCAAATTCACCAGTTt in Y1H-Gold, SD medium –Trp.
4541	Positive control	Y1H Screen p53His2 + pGADRec2-53 in Y1H-Gold, SD medium -Trp/ -Leu
4542	Negative control	Y1H Screen pHis2.1 + pGADRec2-53 in Y1H-Gold, SD medium -Trp/ -Leu
4707	Y1H-Gold	Y1H-Gold strain from ≠3793, Y1H-Screen; 2xYPAD
4696	pBT10GUSd35SLUC	pBT10GUS_d35SLUC from Glycerol ≠4511, *E. coli*, LB

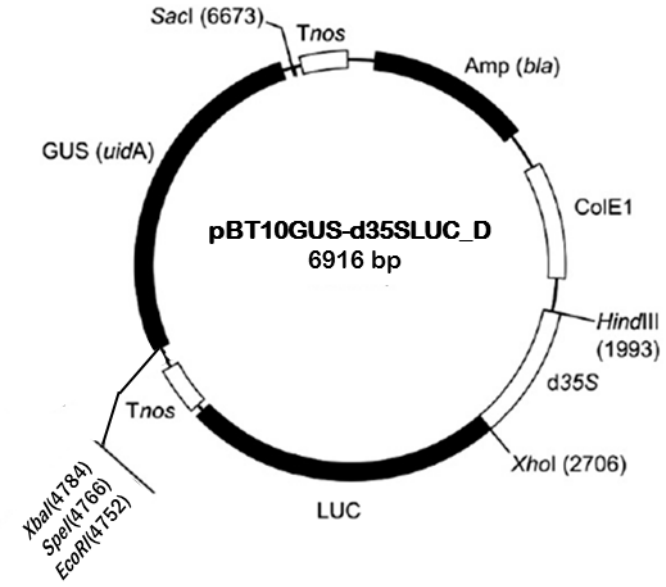
		medium Carb^r
4419	pHis2.1	pHis2.1 in *E. coli* XL1-blue; LB medium Kan^r
3431	pRT103-GUS	pRT103-GUS in *E. coli*, LB medium Carb^r
4017	pORE_O2_d35S_pA	pORE_O2_d35S_pA in *E. coli,* LB medium Kan^r
3433	pBT10LUC	pBT10LUC in *E.coli*, LB medium Carb^r
4383	pORE_O2_d35S_pA_ Cmyc	pORE_O2_d35S_pA_Cmyc in C58C1 strain, LB medium + Carb, Kan, Rifa 50 mg/L
4447	4x30H_8_M1S2_pBT 10LUC	4xSeq24 (4x30H_8_M1S2)_pBT10LUC) in *E.coli*, LB medium Carb^r
4130	4x30H_8_M1S2_pBT 10GUSd35SLUC	4xSeq24 (4x30H_8_M1S2)_pBT10GUSd35SLUC) in *E.coli*, LB medium Carb^r
3836	30I_8_M1S3-pBT10GUSLUC	4xSeq22 (30I_8_M1S3)-pBT10GUSLUC in *E. coli*, LB Carb^r
4646	4xSeq22-pHis2.1	4xSeq22 (30I_8_M1S3) in *E. coli* XL1blue, Kan^r
4647	4xSeq22-pHis2.1	4xSeq22 (30I_8_M1S3) in Y1H-Gold, SD-Trp
4325	WRKY70-pORE-d35S-pA-cmyc	WRKY70_pORE-d35S-pA-cmyc in *A. tumefaciens* C58C1 strain. LB: Carb, Rifa, Kan 50 mg/L.

Vectors

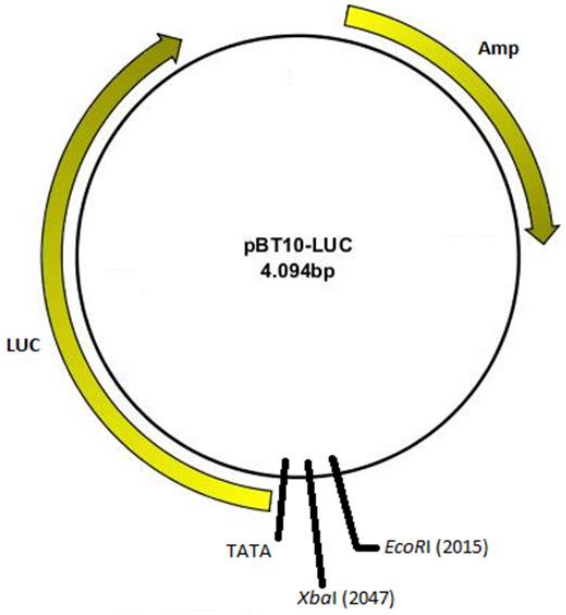
1. pBT10GUS-d35SLUC



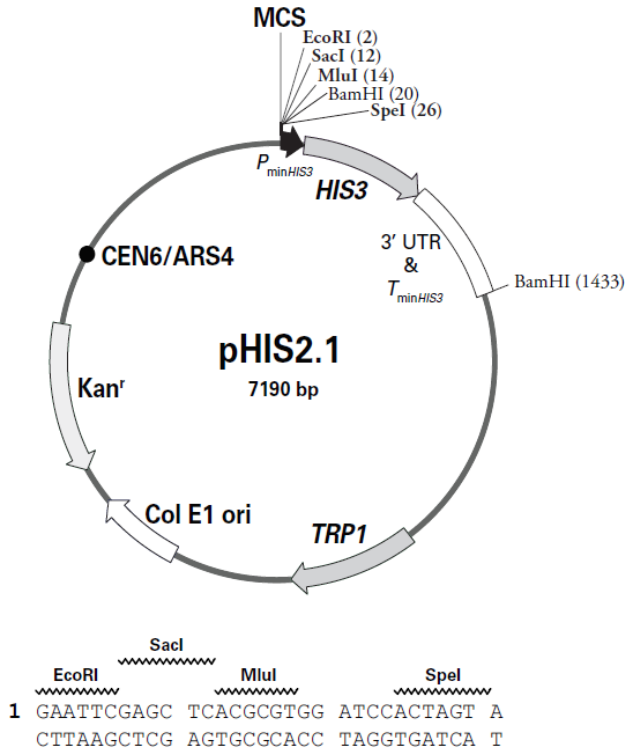
2. pBT10GUS-d35SLUC_D



3. pBT10-LUC

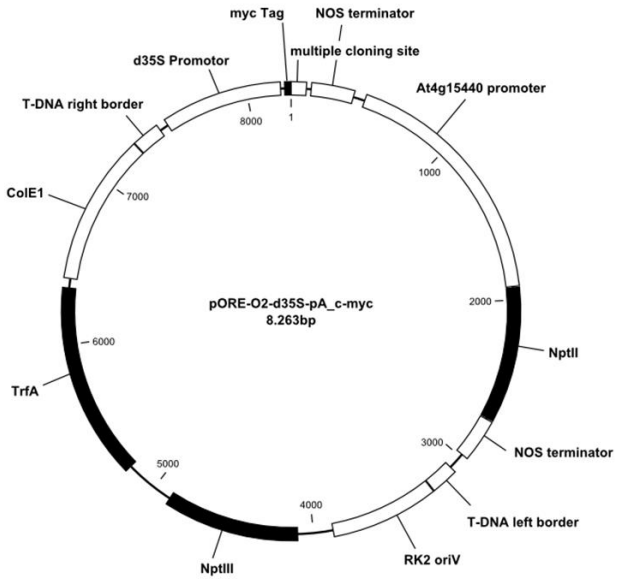


4. pHIS2.1

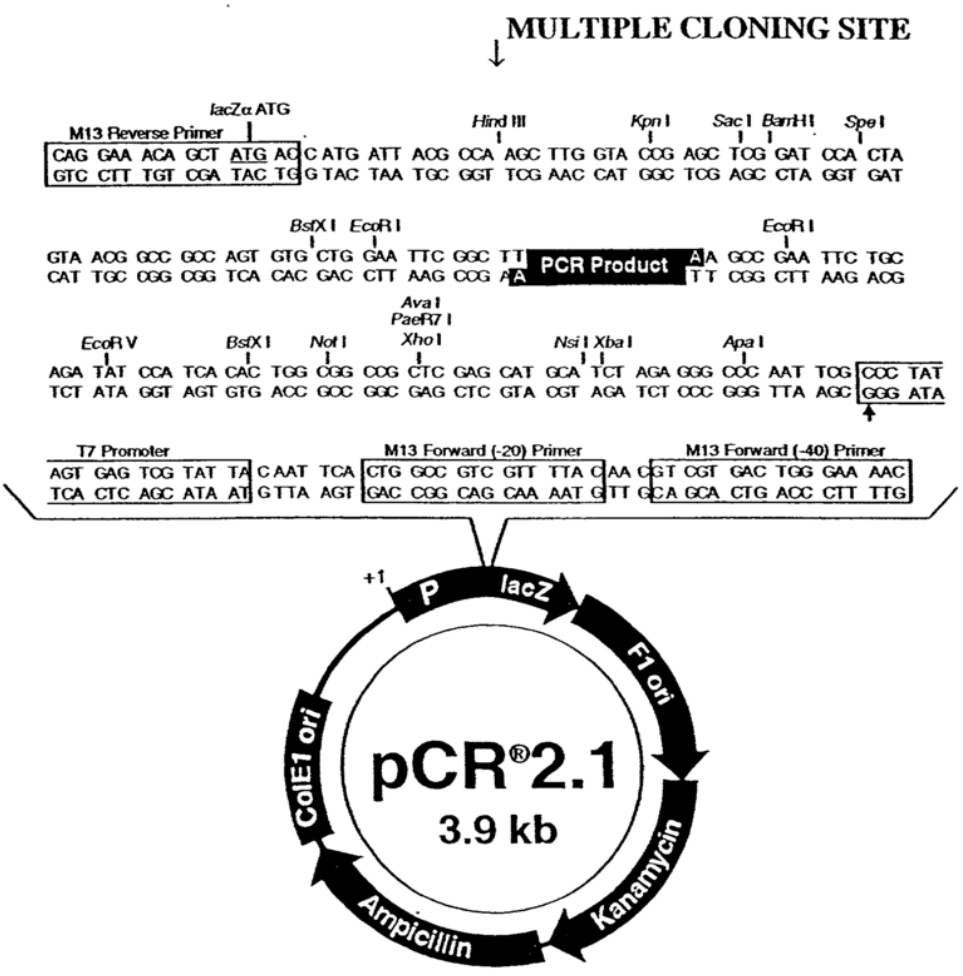


Restriction Map and Multiple Cloning Site (MCS) of pHIS2.1 Vector. Unique restriction sites are in bold.

5. pORE-O2-d35S-pA_c-myc


myc Tag
NOS terminator
multiple cloning site
d35S Promotor
At4g15440 promoter
T-DNA right border
8000
1
1000
ColE1
7000
pORE-O2-d35S-pA_c-myc
8.263bp
2000
NptII
6000
TrfA
3000
NOS terminator
5000
4000
T-DNA left border
NptIII
RK2 oriV

6. pCR2.1


MULTIPLE CLONING SITE
lacZα ATG
M13 Reverse Primer
Hind III
Kpn I
Sac I
BamH I
Spe I
CAG GAA ACA GCT ATG AC C ATG ATT ACG CCA AGC TTG GTA CCG AGC TCG GAT CCA CTA
GTC CTT TGT CGA TAC TG G TAC TAA TGC GGT TCG AAC CAT GGC TCG AGC CTA GGT GAT
BstX I
EcoR I
EcoR I
GTA ACG GCC GCC AGT GTG CTG GAA TTC GGC TT PCR Product AA GCC GAA TTC TGC
CAT TGC CGG CGG TCA CAC GAC CTT AAG CCG AA TT CGG CTT AAG ACG
Ava I
PaeR7 I
EcoR V
BstX I
Not I
Xho I
Nsi I
Xba I
Apa I
AGA TAT CCA TCA CAC TGG CGG CCG CTC GAG CAT GCA TCT AGA GGG CCC AAT TCG CCC TAT
TCT ATA GGT AGT GTG ACC GCC GGC GAG CTC GTA CGT AGA TCT CCC GGG TTA AGC GGG ATA
T7 Promoter
M13 Forward (-20) Primer
M13 Forward (-40) Primer
AGT GAG TCG TAT TA C AAT TCA CTG GCC GTC GTT TTA C AA CGT CGT GAC TGG GAA AAC
TCA CTC AGC ATA AT G TTA AGT GAC CGG CAG CAA AAT G TT GCA GCA CTG ACC CTT TTG
+1
P
lacZ
F1 ori
ColE1 ori
pCR®2.1
3.9 kb
Ampicillin
Kanamycin

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